

HOMEOVISCOUS ADAPTATION IN
TURTLE LUNG SURFACTANT

CENTRE FOR NEWFOUNDLAND STUDIES

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HOMEOVISCOUS ADAPTATION IN TURTLE
LUNG SURFACTANT

A thesis

by

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Submitted in partial fulfillment of
the requirements for
the degree of Master of Science

Department of Biochemistry
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Abstract

To determine if homeoviscous adaptation occurs in lung surfactant of an air-breathing ectotherm so as to maintain a specific physical state critical for function at different temperatures, map turtles were acclimated to 5, 14, 22 and 32°C for at least one month. Lipid analyses were carried out with lung lavage fluid and lung tissue using thin layer chromatography and gas-liquid chromatography.

Whereas phospholipid content per g of fresh lung tissue in lavage fluid was significantly elevated at 32°C as compared to 5 and 14°C, no significant differences were observed in phospholipid content in lung tissue with changing temperature. Lecithin was the major phospholipid in lavage fluid, constituting more than 70% of total phospholipid, and it increased with increasing temperature. The two major phospholipids, in lung, phosphatidylcholine and phosphatidylethanolamine showed changes with temperature and trends which were complementary to each other. There was a proportionate increase in palmitate and decrease in palmitoleate content in both total lipid and phosphatidylcholine in lavage fluid. The changes in palmitate content in lavage fluid were not reflective of a general increase of the corresponding fatty acid in the lipids of lung. A significant increase in saturation was found in total lipid and phosphatidylcholine of lavage fluid, and phosphatidylcholine and phosphatidylethanolamine in lung. Saturated lecithin isolated from total lavage had a fatty acid profile consisting primarily of palmitate, 85 mole %. Saturated lecithins, principally dipalmitoyl lecithin, increased as a proportion of lavage lecithin with increasing body temperature. Thermograms of model lecithin mixtures approximating

the lecithins in lavage from turtles kept at 5 and 32°C showed a shift in transition temperature, implying the difference in dipalmitoyl phosphatidylcholine content in the lipids resulted in a change in physical state. These findings were consistent with the preservation of a constant specific phase(s) in surfactant lipid at each temperature of acclimation.

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Abbreviations

mN/m	milli Newton per metre.
nM	nanometre.
w/w	weight/weight.
v/v	volume/volume.
mg	milligram.
µg of P	microgram of phosphorus.
g	gram
°C	degree Celcius.
Pi	inorganic phosphate.
DPPC	1,2-dipalmitoyl- <u>sn</u> -glycero-3-phosphocholine.
CDP	cytidine diphosphate
CTP	cytidine triphosphate
[U- ¹⁴ C]-glucose	uniformly labelled glucose.
PC	phosphatidylcholine.
PE	phosphatidylethanolamine
PG	phosphatidylglycerol.
PS	phosphatidylserine.
PI	phosphatidylinositol.
SM	sphingomyelin.
P (in tables of significant changes)	probability

Introduction

1. History

According to Goerke (1974), credit for the earliest observation of mammalian lung surfactant probably should go to Frederic Marten (1671), who under the heading "How they catch the whale", recorded the following: "When the whales blow up the water, they fling out with it some fattish substance that floats upon the sea like sperm, and this fat, the Malle-mucks devour greedily ...". Assuming the fattish substance to be lung surfactant, it would seem that the lipid nature of lung surfactant has long been anticipated. However, experimental events which led to the elucidation of lung surfactant did not take place until 1929 when the importance of surface forces in respiratory mechanics, especially during lung retraction was recognized. But, as recently as the 1950s, most pulmonary physiologists believed that recoil of the lung in expiration originated in elastic fibers that had been stretched during inspiration. The important experiment by von Neergaard (1929) seemed to have been forgotten.

To determine the contribution of surface tension to the retractile force of the lung, von Neergaard (1929) constructed pressure-volume curves of an excised lung filled first with air and then with an isotonic solution of gum arabic. In the latter case where the air-alveoli interface was eliminated, retraction was due only to the tissue elasticity. A measure of surface tension at a given lung volume was obtained by subtracting the pressure for solution-filled lung from that of the air-filled lung at the same volume. It was found that the surface tension in the alveoli contributes $2/3$ to $3/4$ of the retraction

force of the lung. In his report, he recognized that unrestrained surface forces could cause alveoli to collapse and stated his belief that surface tension in alveoli was of vital importance in the expansion or collapse of lungs of newborn animals. He even suggested that the surface tension of alveoli was reduced by the accumulation of surface active substance, surfactant. Unfortunately, the significance of this work was overlooked for more than twenty years.

Then, in 1954, Macklin postulated that the mucoid film overlying the epithelium of the lung alveoli was produced by what he called granular pneumonocytes. This film, he observed, might be responsible for maintaining constant surface tension of the alveolar wall and might be causally related to the myelin figures emerging from the surfaces of alveolar walls when sections of fresh lung were mounted in aqueous solution (Macklin, 1954).

That same year, Radford calculated the surface area of the lung by using the concept that the total free energy in a surface under constant tension is equal to the product of the surface area and the surface tension. Free energy can be derived from the area beneath the volume-pressure curve of the lung and he assumed the surface tension of alveoli to be equal to that of serum, 50 mN/m. To his amazement, the value he obtained for surface area of the lung was only one tenth of the anatomic measurements (Radford, 1954). He commented that the discrepancy could only be accounted for if the lung surface consisted of a highly surface active substance. His work attracted much attention and served to rekindle the interest of lung physiologists in the subject of surface tension.

The year

^ 1955 saw another important event in the history of lung surfactant

research occur. Pattle observed that the foam produced during pulmonary edema was stable towards antifoaming agents whereas froth from serum was not. Since the foam disappeared in air-free water, he inferred that the stability of the foam bubbles was not due to the impermeability of their surfaces to air but rather to the fact that the surface tension of the foam bubbles was zero or near zero. These findings taken together suggested to him that the surface layer of his stable bubbles could have come from the depths of the lung where the mucous layer was secreted to abolish surface tension (Pattle, 1955).

The unfolding of the fascinating story about lung surfactant was accelerated by the introduction of the Langmuir-Wilhelmy balance to measure surface tension of saline extracts from homogenates of mammalian lung tissue (Clements, 1957). It was found that the surface tension of the extracts decreased from 46 to 10 mN/m as surface area was compressed. Clements (1957) made the crucial observations that surface tension was high at high lung volumes and dropped to very low values when lung volume decreased, thus preventing the alveoli from collapsing. This phenomenon was due to the surface active material in the lung. In the following years, Clements, Brown and their coworkers formulated the theory of alveolar surface tension and alveolar stability (Clements et al., 1958; Brown et al., 1959; Clements et al., 1961) based on the principle that pulmonary surface tension varies with surface area and thus with alveolar volume. These theoretical considerations have helped to usher in a new era of research on lung surfactant, which witnessed hundreds of manuscripts dealing with the chemistry and physiology of lung surfactant being published each year. From these works, a more detailed picture of lung surfactant has emerged.

(a) Morphology: The term "surfactant system of the lung" was coined by Scarpelli (1968) to connote an extracellular lining layer between the wall of the alveolar cells and the air in the lung. It consists of a monolayer of phospholipid and a subphase of liquid containing proteins, carbohydrates and osmiophillic "tubular myelins" which are formed by deposition of phospholipid (Weibel and Gil, 1968; Dermer, 1969). The thickness of this alveolar lining is about 16-20 nm in freeze-etched specimens from mammalian lungs (Roth et al., 1973). Type II cells are believed to be responsible for the manufacture and secretion of the surface active material (Macklin, 1954; Klaus et al., 1961; Buckingham et al., 1964) which exists in the cell as lamellar bodies (Gil and Reiss, 1973). It has been suggested that the stress on the secreted lamellar bodies as they enter the extracellular fluid causes them to fold into a zig-zag pattern to form tubular myelin, (Sanderson and Vatter, 1977) which then spread at the air liquid interface to establish a monolayer. This final process is thought to be induced by divalent calcium ions in the subphase whose sites of interaction are within the lamellar bodies (Paul et al., 1977). A diagrammatic representation based upon that of Goerke (1974) is shown in Fig. 1 .

(b) Composition: Surface active materials purified from lung lavage have the following composition (in % dry weight): protein 11, lipid 88, hexose and hexosamine about 1 (King and Clements, 1972b).

(i) Lipid: There is much similarity in the kinds of lipids found in mammalian pulmonary surfactant regardless of isolation techniques or choice of animal species, (King and Clements, 1972b; Pruitt et al., 1971; Abrams, 1966; Frosolono et al., 1970). Similarly the composition is

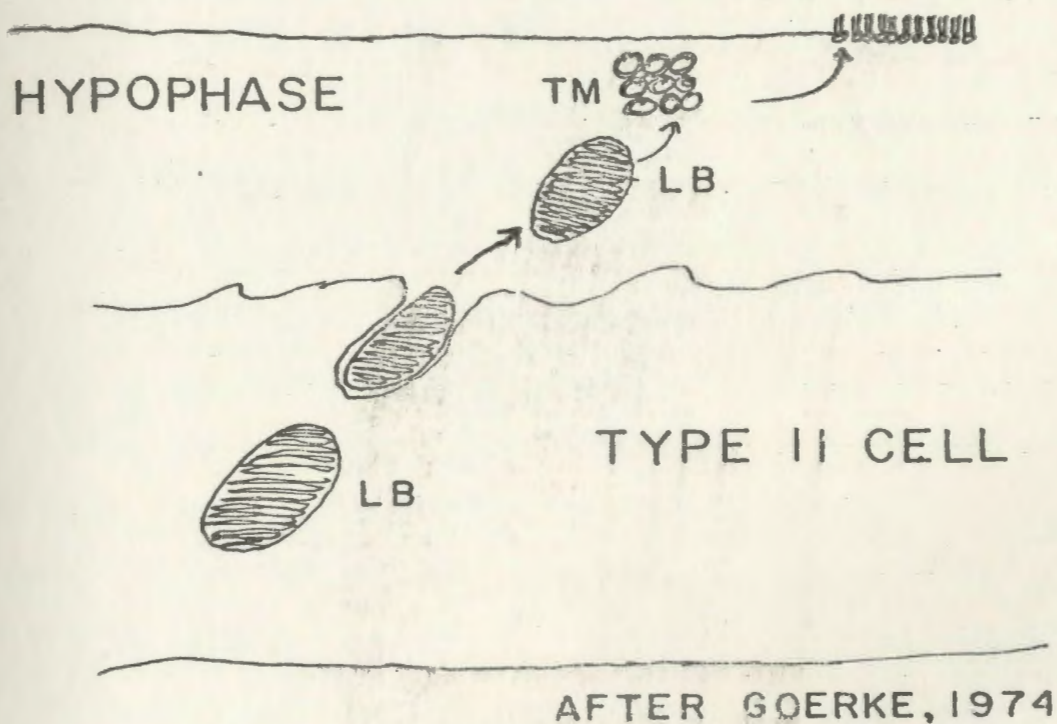


Figure 1: Diagrammatic representation of lung surfactant. LB: Lamellar bodies; TM: Tubular myelin. Arrows indicate the possible stages the lipids undergo after secretion.

independent of whether the material is of extracellular or intracellular (lamellar bodies) origin. Phospholipids predominate and constitute about 85-90% (w/w) of total lipid. Neutral lipids, such as cholesterol, triglycerides and free fatty acids make up the remainder. 74-85% by weight of phospholipid is lecithin and the remainder is composed by phosphatidylethanolamine, phosphatidylglycerol and sphingomyelin (King et al., 1973; Morgan et al., 1965; Pflieger and Thomas, 1971). Dipalmitoyl phosphatidylcholine stands out as the principle constituent, accounting for 45% of the total lipid by weight, (Clements and King, 1976) or 24-36 mole % of total lipid (see e.g. Träuble et al., 1974). Small amounts of acidic phospholipids have also been detected, especially in association with a hydrophobic apoprotein of surfactant (King et al., 1973).

(ii) Protein: While the lipid fraction of lung surfactant with its unusually high content of DPPC (Mason, 1976) has been a subject of intense investigation and much is known about its composition and physiological functions, the problem of the existence of the protein(s) unique to lung surfactant remains unresolved. The view that lung surfactant consists of lipoprotein was first held by Abrams (1966) and several investigators have isolated surface active materials which contain proteins (King and Clements, 1972a; Pawlowski et al., 1971; Pruitt et al., 1971; Galdston et al., 1969; Sueishi et al., 1977). In the meantime, experiments that argued against the existence of specific proteins were also reported (Scarpelli et al., 1967; Scarpelli and Colacicco, 1970, Shelley et al., 1975).

It was King and coworkers (King et al., 1973, 1974) who first isolated and identified the apoprotein from sodium dodecyl sulphate-

treated canine surface active material. Two proteins were reported - at 11,000 daltons and 34-35,000 daltons (King et al., 1973, 1974). The 11,000 dalton apoprotein is comparable in hydrophobicity to proteins found in membranes and is therefore thought to be embedded in lipid matrices. The immunological properties of lung surfactant appear to be determined principally by the antigenicity of the 34,000 dalton apoprotein (Clements and King, 1976). By latex agglutination immunoassay, King et al. (1975) were able to show that the two apoproteins first appeared in human amniotic fluid at about 32 week gestation and their concentration was approximately paralleled by the change in surface active lipids. It is possible that the 11,000 dalton protein is a catabolic fragment of the 35,000 dalton apoprotein (King, 1977).

The 35,000 dalton protein from canine lung surfactant of King et al. (1974) is presumably the same as the 36,000 dalton protein found by Sawada and coworkers (1977) to be the main apoprotein of bovine lung surfactant material.

In spite of the impressive data collected on apoproteins in surfactant, the specificity of these proteins to lung surfactant remains to be absolutely confirmed. Doubts were raised by at least two recent reports, which indicated that there was no specific protein associated with surfactant phospholipid obtained from saline lavage fluid of the lung (Maguire et al., 1977; and Colacicco et al., 1977).

(iii) Carbohydrates: Early studies which were done with indirect methods on crude materials (Pattle, 1955; Brown et al., 1959) seemed to indicate the presence of polysaccharides in surface active preparations, but the relationship of intra-alveolar carbohydrate to surfactant remains unclear. Gel filtration of lung lavage fluid yields a fraction that

contains glucose, galactose, galactosamine and fucose identified by qualitative tests (Scarpelli et al., 1967; King et al., 1975) together with phospholipid. Since this fraction emerges in the void-volume of the column, the physical relationship of carbohydrate to phospholipid components is uncertain. So are the hexose and hexosamine which have been found in fractions of surface active material prepared by gradient centrifugation (King and Clements, 1972b). However, the carbohydrates which migrate with the 11,000 dalton apoprotein in polyacrylamide gel electrophoresis are probably covalently bound to it (King et al., 1973). As discussed above, the specificity of this apoprotein and the carbohydrate attached to it awaits further confirmation.

Two glycopeptides have been isolated in lung lavage fluid (Passero et al., 1973) and in preparations of lamellar bodies in alveolar type II cells from patients with alveolar proteinosis (Passero et al., 1974). The significance of these findings in relation to the nature, quantity and attachment of carbohydrates to proteins in lipids in normal lung surfactant is a subject for further exploration.

2. The Physiological Role of Lung Surfactant in Respiration

To help to appreciate the importance of this thin alveolar lining in the lung, a brief discussion of the effects of surface tension in an air-water interface will be undertaken.

Molecules in the bulk of liquid, experience intermolecular attractions called cohesive forces and what makes the molecules at the surface unique is the imbalance of those forces in the air-liquid interface, such that there is a net force which tends to pull the surface molecules towards the centre of the bulk. The measure of this tendency is termed surface tension which is expressed in the unit of mN/m.

Curved surfaces with such air-liquid interfaces would experience a contracting force to minimize the surface area, which is related to the difference in pressure across the surface according to Laplace rule (1807)

$$P = T \left(\frac{1}{r_1} + \frac{1}{r_2} \right)$$

where P is difference in pressure across the curve surface(s).

T is the total tension in the surface(s).

r_1 and r_2 are the principal radii of curvature of the surface(s).

For a spherical segment $r_1 = r_2 = R$

then $P = \frac{2T}{R}$

If the situation is such that there is only one air-liquid interface,

$$T = \gamma$$

where γ = surface tension.

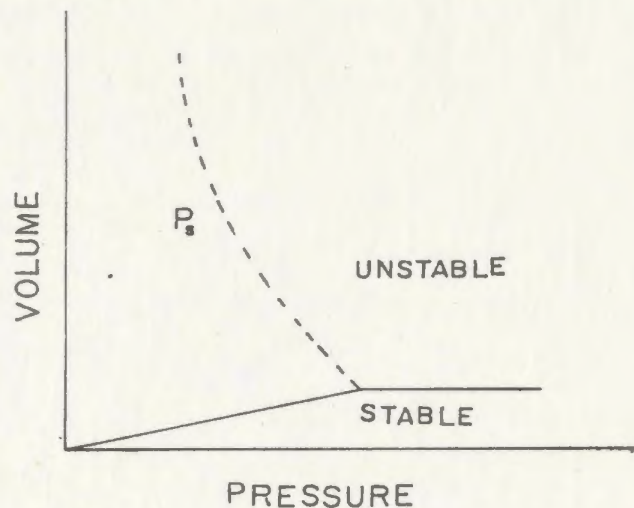
Therefore, we get $P = \frac{2\gamma}{R}$ which is applicable to a bubble with only one

surface.

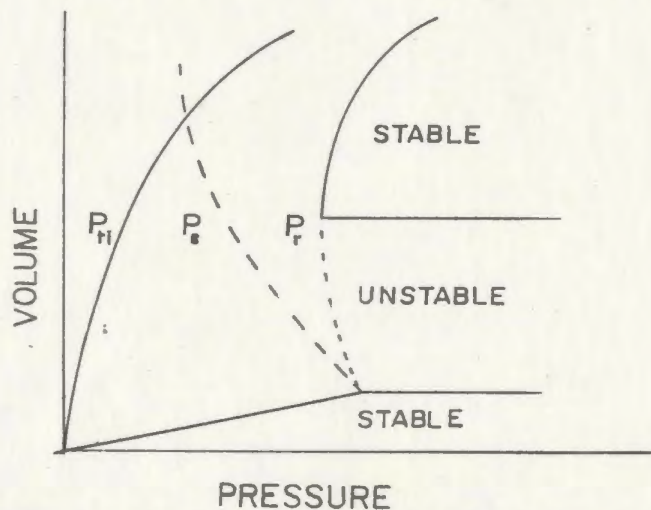
If we accept the model of lung alveoli as bubbles at the end of a tube (airway), it can be seen that when the bubble increases in size as shown in Fig. 2a, the radius of curvature decreases from infinity to R when the bubble assumes the shape of a hemisphere. According to the formula, if the surface tension remains constant, pressure would be highest at this point, as in the volume-pressure curve in Fig. 2b. So long as the bubble does not exceed the hemispherical shape, it is stable, as indicated by the positive slope of the curve, that is stability is conferred by its geometry. However when it exceeds the volume of that of a hemisphere, R increases, resulting in a decrease in pressure, the



- a. Alveolus at the end of airway .
Radius decreasing during inflation.

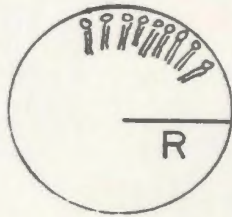


- b. Volume-pressure diagram to show the condition of the bubble-like alveolus when surface force P_s is acting alone (see text).



- c. Volume-pressure diagram showing the resultant force in the bubble-like alveolus (see text). P_s = surface force, P_{ti} = tissue force, P_r = resultant force.

Figure 2: Diagrams to demonstrate mechanics of an alveolus considered as a bubble.



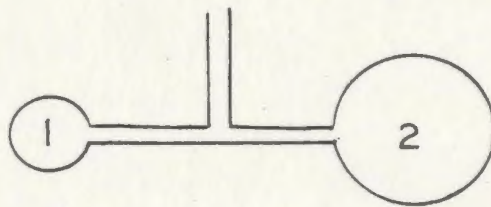
$$\Delta P = \frac{2\gamma}{R}$$

WHERE ,

ΔP = EXCESS PRESSURE

γ = SURFACE TENSION

R = RADIUS



γ CONSTANT

$$\Delta P_1 > \Delta P_2$$

1 COLLAPSES INTO 2

γ VARIES WITH R

$$\Delta P_1 = \Delta P_2$$

BOTH STABLE

Figure 3: Instability of lung due to varying sizes of alveoli and the condition for stability.

plot of this part of the curve appears in the dashed line in Fig. 2b. Since stability demands a positive compliance, i.e. $\frac{dv}{dp} > 0$, this portion of the curve is therefore unstable. Nevertheless, it has been shown that tissue force also confers stability to the alveoli which would again be stabilized at high volumes (Fig. 2c) (Mead, 1961). The unstable region of the resultant curve is defined by the part having a negative slope, i.e. -ve elasticity or compliance.

Clements (1957) pointed out that a low coefficient of surface film compressibility, which is expressed in the sharp fall of tension during deflation of lung would have a stabilizing effect on the alveoli if the film is present in the air space. This lowering of surface tension as the alveoli deflates is effected by lung surfactant, allowing the alveoli to remain open over the region labelled "unstable" in the graph (see, e.g. Clements and Tierney, 1965).

It is well known that individual alveoli are independent in their expansion and contraction (Radford et al., 1954; Radford and McLaughlin, 1956). Hence in the retracting lung during exhalation, alveoli of varying sizes are expected. Fig. 3 is a simplified representation of what may happen during expiration. If surface tension in the small alveoli were to be the same as that of the big ones, the pressure across the small alveoli would be higher than that in the big ones, and there would be a tendency for the smaller alveoli to empty into the larger ones, which in eventuality would result in the collapse of the lung. However, the presence of lung surfactant lowers the surface tension of the smaller alveoli to a greater extent than that of bigger ones and thus stabilizing the lung volume at functional residual capacity, which is

about 40% of the maximum volume

(Clements et al.,

1961).

Direct determination of surface tension in the lung showing that surface tension remained below 9 mN/m for at least 30 minutes (Schürch et al., 1976) has dispelled doubts about the stability theory and the necessity of lung surfactant to stabilize the alveoli (Fung, 1975; Reifenrath, 1975).

The consequences of the absence of pulmonary surfactant are observed in the lungs of newborn infants who show respiratory distress syndrome. Avery and Mead (1959), using a modified Langmuir-Wilhelmy balance to examine the surface properties of saline extracts of lungs from infants, observed that surfactant was present in the lungs of mature newborn babies but deficient in the lungs of very small premature infants and infants dying of hyaline membrane disease. It was also found that excised lungs from diseased infants were nearly airless at pressures that would be the equivalent of end-expiratory pleural pressures of 1-2 cm of H_2O whereas normal lungs contain about 40% of total lung volume at these pressures (Gribetz et al., 1959). These observations are consistent with the theory that, as a result of the incapability of immature lung to synthesize or secrete lung surfactant into the alveolar surface, the lungs do not retain air during deflation and collapse after the first breath (Avery et al., 1973; Avery and Fletcher, 1974). Continued effort as great as the initial one, which amounts to a pressure of 20-30 cm of H_2O (Gruenwald, 1963) is required to open up the alveoli with each successive breath. If untreated, the infants die of exhaustion, lack of oxygen and mechanical damage, among other things.

3. Dipalmitoylphosphatidylcholine

While saturated lecithin was known to be present in lung for some time (Thannhauser et al., 1946; Popjak and Beekmans, 1950), DPPC was first suggested to be the surface tension lowering component in saline extracts of mammalian homogenates by Klaus and coworkers (1961) and actually demonstrated in preparations with low surface tension by Brown (1964).

It has since been confirmed that films of DPPC are able to sustain extremely high surface pressure (π) ($\pi = \gamma_0 - \gamma$, where γ_0 is the surface tension of the pure liquid subphase and γ is the surface tension when a monolayer is spread on the surface), of the order of 70 mN/m at maximum film compression on a saline subphase. This corresponds to surface tension of about 2 mN/m or less. By comparison long chain fatty acids and alcohols, which are also known to be surface active, can only lower surface tension of water to 30 mN/m at maximum compression (Notter and Morrow, 1975). Not all saturated phospholipids are as effective as DPPC in lowering surface tension. Dipalmitoyl phosphatidylethanolamine films were found to reach a collapsing surface pressure of 55 mN/m, corresponding to surface tension of 17 mN/m (Tabak and Notter, 1975). Since the temperature at which these studies were carried out was not specified, it was perhaps reasonable to assume that the results were obtained at room temperature.

Since the "strength" of a surfactant is described as the capacity to decrease tension, it is clear that stability of the surface film at high pressure is actually the determinant of such "strength" (Clements and Tierney, 1965). A measure of stability can be obtained by spreading a surface film at an air-liquid interface and then decreasing its area

until surface tension is very low. At constant areas the tension increases towards equilibrium, which is 24-28 mN/m for DPPC, as the film is metastable and material escapes from the interface (Tierney, 1965). The rate constant of such a process which follows approximately first-order kinetics can be calculated (Clements, 1967). The value for DPPC at 37°C is very low, $K' < 0.006 \text{ min.}^{-1}$, showing that DPPC is very stable in this statically compressed state (Clements, 1977). Unsaturation in the hydrocarbon chains or decreased polarity of the functional groups of the phospholipid gives a less stable film (Clements and Tierney, 1965). Admixture of less polar material with strongly polar lipids reduces stability (Tierney and Johnson, 1961). Also surface films of 1-palmitoyl-2-myristoyl-sn-glycero-3-phosphocholine (PMPC) at 37°C collapse about 40 times as fast as DPPC measured by the two indices of collapse rate: $(d\gamma/dt)A$ and $(d\ln A/dt)\gamma$ where t = time, γ = surface tension and A is area of the film surface (Goerke and Clements, 1973).

It is essential that DPPC films should possess two properties to be useful as lung surfactant. These are an ability to lower surface tension to a low value and to remain stable at such low tension. Then, during exhalation, the alveolar surface tension will be sufficiently low to stabilize the lung, and it does not increase to values that would cause atelectasis at normal and expiratory transpulmonary pressures.

There is evidence to show that dynamic compression of DPPC films imparts a metastable structure, a state when compression produces surface tension lower than the equilibrium values of the film, which is distinct from the static molecular structure.

The data suggested that multilayers are formed when films are highly compressed, which inhibits relaxation processes and causes the DPPC film to become more stable (Tabak et al., 1977). This unique behaviour of DPPC is important, as lung surfactant may exist in such a state over a significant part of the breathing cycle (Clements et al., 1970).

Finally, of the several surface active phospholipids present in lavage fluid obtained by pumping saline in and out of a mammalian lung, only DPPC is present in sufficient quantity to make a monolayer over the entire surface of the lung (Tierney, 1974).

4. Metabolism of Lung Surfactant

Only in the last decade or so has it been widely appreciated that the extremely complex mechanics of the lung are dependent upon its metabolism and that the exocrine secretion of the lung, including lipids, proteins and possibly carbohydrates is vital for survival. With the rediscovery of lung surfactant and its putative functions, it is natural that much of the research efforts have been directed to the biosynthesis, elaboration and secretion of lipid constituents of the surfactant system of the lung, chiefly that of DPPC. A number of relatively recent reviews are available in this area (see, e.g. Frosolono, 1977; Mason, 1976) so that only the main points about metabolism will be discussed here together with pertinent information appearing since these reviews.

Much of the information accumulated derives from work performed on the whole lung which is of considerable cellular diversity. Over 40 different cell types are present in the mammalian lung (Sorokin, 1970) and it is only reasonable to assume that each cell type does not con-

tribute equally to the metabolic process. To make things worse, type II cells which are known to synthesize and secrete lung surfactant (Macklin, 1954; Buckingham et al., 1966; Askinr and Kuhn, 1971; Chevalier and Collet, 1972) constitute only 14% of parenchymal lung cells (Weibel et al., 1976). Hence, it is hardly surprising that some of the crucial evidence presented below demands verification and confirmation. However, a very important step has been taken by Kikkawa and Yoneda (1974) who reported the first method for isolating type II cells, thus promising direct evaluation of the metabolism of lung surfactant. Now it is also possible to grow human mammalian type II cells in culture (Avery, Personal Communication) which should help greatly to elucidate problems of surfactant lipid metabolism.

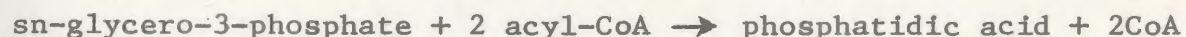
Synthesis: (a) Source of fatty acids: Although there is a significant uptake of fatty acid from the circulation, the blood flow through the lungs necessary for gas transport far exceeds the requirement of providing substrate for metabolism within the lung (Naimark, 1973). Uptake of fatty acid is probably not rate limiting nor selective for palmitate but is dependent on the concentration of fatty acids in the perfusate and is altered by the distribution of pulmonary bloodflow (see, e.g. Mason, 1976).

Another source of fatty acid comes from de novo biosynthesis in the lung, which takes place primarily in the cytosol, (Schiller and Bensch, 1971) producing palmitate in large part (Gross and Warshaw, 1974). Fatty acids are elongated in lung mitochondria but the de novo pathway utilizing malonyl-CoA may also be present (Tombropoulos, 1964).

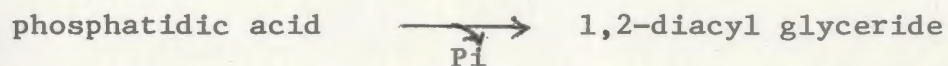
(b) Biosynthesis of phosphatidylcholine:

(i) Cytidine diphosphate choline (CDP-choline pathway): In the

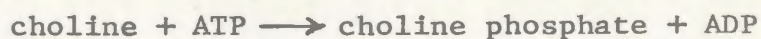
lung, the biosynthesis of phosphatidylcholine takes place primarily through the CDP-choline pathway (Kennedy and Weiss, 1956), which involves the following steps: (1) Formation of phosphatidic acid through esterification of sn-glycero-3-phosphate (Mims and Zee, 1971; Mims and Kotas, 1973; Moriya and Kanoh, 1974; Frosolono et al., 1975):



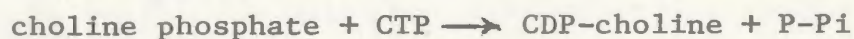
Acylation of dihydroxyacetone phosphate is also possible. However, the ultimate source of sn-glycero-3-phosphate is glucose (Gassenheimner et al., 1972). The acyltransferases which catalyses this reaction are present in the endoplasmic reticulum (Hendry and Possmayer, 1974). (2) Production of 1,2-diacyl glyceride from phosphatidic acid:



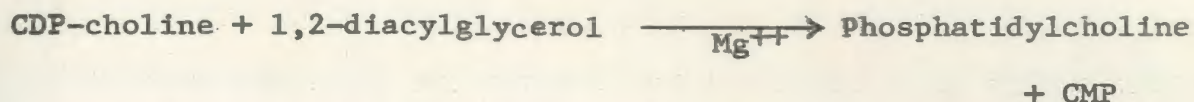
The enzyme, phosphatidic acid phosphohydrolase has been found in the perilamellar surface of lamellar bodies in hamster type II cells using electron microscopic histochemical technique (Meban, 1972). Very high specific activity of this enzyme is also found in lamellar bodies isolated from pig lung (Spitzer, 1975). (3) Incorporation of choline, which involves these sequential enzymatic reactions: (a) phosphorylation of the free base catalyzed by choline kinase:



(b) conversion of choline phosphate to CDP-choline by choline phosphate cytidyltransferase:



(c) transfer of phosphorylcholine to the 1,2- acyl diglyceride by choline phosphotransferase:



These enzymes have been found to reach full activity in fetal rat lung at about the time of birth (Artom, 1968). Measurements of specific activities of the three enzymes have been made in lungs of monkey (Farrell, 1973) and rat fetuses (Weinhold et al., 1973; Farrell et al., 1974).

In most biological phospholipids, there is an equimolar amount of saturated and unsaturated fatty acids in the positions 1 and 2 of the glycerol backbone respectively. The DPPC molecule is unusual in that it has two saturated fatty acids, palmitates, esterified to it. Obviously, the lung must have some special mechanism(s) for the synthesis of the unique phospholipid. At least two major routes are possible. (a) The initial acylations of sn-glycerol-3-phosphate may be highly selective so that large amounts of dipalmitoyl phosphatidic acid are produced. This acyl distribution pattern would be maintained throughout the subsequent steps to the phosphatidylcholine level. (b) The phosphatidylcholine first produced has the "usual" pattern (i.e. glycerol-1-saturated and 2-unsaturated residues). This phosphatidylcholine would then be modified to give DPPC through the interaction of other enzymatic reacylation systems.

There is evidence that mechanism (a) can be responsible for some DPPC but it cannot be the primary pathway (Vereyken et al., 1972; Possmayer et al., 1977a). It has also been shown that choline phosphotransferase does not exhibit substrate selectivity toward 1,2 diacylglyceride species (Moriya and Kanoh, 1974). As shown by Rooney and

Wailee (1977) it even exhibits preference for unsaturated diacylglycerols. For mechanism (b), two pathways are possible: (i) Deacylation and reacylation of phosphatidylcholine via the pathway proposed by Lands (1958): lyso PC + acyl CoA \longrightarrow PC + CoA-S. Lands (1958, 1960) and Webster (1965) were the first to show that there was significant lyso-phosphatidylcholine acyltransferase activity in the lung. Studies on Type II cell adenomas in mouse lung point to the importance of lyso-phosphatidylcholine acyltransferase in the biosynthesis of DPPC (Snyder and Malone, 1975). In studying synthesis of lecithins in isolated type II cells, Smith and Kikkawa (1978) reported evidence which suggested that this is the major pathway of the type II cell as compared to the second possible route (ii) the transacylation pathway between two lysolecithin molecules first discovered by Erbland and Marinetti (1965)



It has been shown that there are marked increases in enzyme activities of lyso PC pathways (1) and (2) with gestational and postnatal ages in rabbit lung (Tsao and Zachman, 1977). The activity of lysophosphatidylcholine: lysophosphatidylcholine acyltransferase increased dramatically to a maximum at one day before birth, implying a sharp rise of saturated PC in the perinatal periods close to birth is mainly attributable to transacylation pathway (Okano and Akino, 1978). There are other reports which argued for the importance of this pathway (e.g. Akino et al., 1971) as well as against it (e.g. Abe et al., 1974).

Experiments on type II cells will certainly shed light on this controversy in the immediate future and a definitive conclusion can soon be expected.

Lecithin can be synthesized not only via CDP-choline but also by

sequential methylation of phosphatidylethanolamine (Bremer and Greenberg, 1959). The products of the reaction, S-adenosyl homocysteine and the N-methylated phospholipids, were identified in lung by Morgan et al., (1965) and the enzymes were found in lung microsomes (Morgan, 1969). Gluck and associates (1967, 1972) have confirmed the presence of the pathway in rabbit and human lung and compared the methylation and CDP-choline pathways in the developing fetus. They found that, while CDP-choline incorporation declined during fetal development, methylation reaction had peak incorporation coinciding with viability of fetal rabbit. The activity of the methyltransferase and methionine activating enzymes in human and rat lung and liver has also been studied (Zachman, 1972). An increase in fetal rat lung phosphatidyl methyltransferase activity was observed in the last 24 hr of gestation, but there was no increase in activity of the same enzyme in human lung with advancing gestation.

Much of the importance given to this pathway relied upon the observation of the identification of phosphatidyl dimethylethanolamine (PDME) in the lung, but the lipid presumed to be PDME in mammalian lung was later shown to be phosphatidylglycerol (Pfleger et al., 1972). The importance of this pathway has since been questioned, and as compared to the CDP-choline pathway, its quantitative contribution in fetal and adult lung is believed to be relatively small (Farrell and Morgan, 1977; Frosolono, 1977).

Degradation: This is one area of lipid metabolism that has received little attention. No systematic study of degradative mechanisms in the lung is found in literature, and consequently, the information discussed below is, at best, fragmentary.

Most studies with radioactivity labelled palmitate, glucose or glycerol suggest DPPC has a biological half-life of 9-16 hrs (Tierney et al., 1967; Thomas and Rhoades, 1970; Spitzer and Norman, 1971; Toshima et al., 1972; Naimark, 1973; Jobe, 1977). An enzyme needed for degradation (but also for the deacylation and reacylation mechanism), calcium ion-dependent phospholipase A₂ has been found to be highly active in lung microsomes (Garcia et al., 1975). Alveolar macrophages are known to be very active in phagocytosis (Myrvik et al., 1961a, b) and later studies revealed that a very potent lysophospholipase activity is present in alveolar macrophage homogenates which may be responsible for the removal of DPPC (Scarpelli, 1968; Weibel, 1967; and Frosolono et al., 1973). There was also the suggestion that phospholipase A systems could be present at the surface of epithelial cells in fetal, and probably adult lamb (Scarpelli et al., 1975). Using the ultrastructural immunoperoxidase method, a dense reaction product of specific antibody against a protein from purified saline lavage of rabbit lung, was detected in phagocytic vacuoles in alveolar macrophages (Sueishi et al., 1977). It thus appears that alveolar macrophages are also related to the clearance mechanism of the protein component of surfactant.

Regulation: In fetal lungs, there is a considerable body of evidence to show that surfactant synthesis is accelerated by the injection of glucocorticoids (see, e.g. Strang, 1977). Liggins (1969) was the first one to suggest that dexamethasone would induce the production of surfactant. The results were confirmed and extended by Kotas and Avery (1971) in rabbits. A four-fold increase of surfactant production at late gestation period of fetal lamb in response to dexamethasone was demonstrated (Platzker

et al., 1975). Glucocorticoid-specific receptors have been discovered in cytoplasm and nuclei of lung cells (Ballard and Ballard, 1972; Giannopoulos et al., 1972) so that the mechanism of action of glucocorticoids in the lung can be depicted as follows: Steroid hormones freely cross the cell membrane and bind to a specific cytoplasmic receptor protein. Interaction of this complex(es) with specific genomes within the nucleus stimulates mRNA synthesis which codes for synthesis of proteins required for DPPC synthesis (Ballard et al., 1977; Ballard, 1977).

As a result of increased plasma corticosterone, the following sequential events have been observed in fetal rat: (a) increased lung choline kinase. (b) increased lung choline phosphotransferase (Farrell et al., 1974); (c) accelerated conversion of choline to lung lecithins (Farrell and Blackburn, 1973); and (d) increased total lung lecithin concentration (Weinhold and Ville, 1965). These findings if temporally connected, appear to support the hypothesis that corticosteroids establish the in vivo timing of enhanced lecithin formation (Farrell and Morgan, 1977). In an extensive review on regulation of pulmonary alveolar development, Taeusch and Avery (1977) summarized an impressive list of findings by suggesting that endogenous cortisol release is a sufficient stimulus to affect the production of fetal surfactant. However, there is evidence to show that the effect of glucocorticoid in fetal lung is to produce an overall increase in phospholipid metabolism rather than a specific increase in phosphatidylcholine synthesis (Possmayer et al., 1977b). As these authors pointed out, their failure to detect increases in the activities of some of enzymes reported by other researchers may be related to the use of whole lung. The availability of type II cell culture will undoubtedly facilitate future

studies on hormone induction and regulation of lung surfactant.

5. Temperature Induced Physical Phenomena of Biological Phospholipid

Phospholipids in water undergo a change in structure from one of ordered packing, the gel phase, to one of high molecular motion, the liquid crystalline phase, when the temperature is increased. The temperature at which this transition occurs is dependent upon the nature of the hydrocarbon chains, the polar region of the molecule, the amount of water present and sometimes (up to a limiting value) the solutes dissolved in water (Chapman, 1973, 1975).

It has been observed that phospholipid films can form expanded (corresponding to liquid crystalline phase in bilayers) or condensed (corresponding to gel phase in bilayers) monolayers depending on the temperature, the length of acyl chains and extent of film compression. An addition of two methylene groups to each chain is approximately equivalent to lowering the temperature by about 20°C, with regard to the physical behaviour of the phospholipid (Phillips and Chapman, 1968).

The importance of phase(s) in the integrity of biological membranes is indicated by the following observations: Yeast cells grown anaerobically cannot synthesize unsaturated fatty acids, under these conditions the phospholipids contain fatty acids of short chain length (carbon number equal to 10, 12 and 14) at the 2 position where unsaturated fatty acid are usually located (Meyer and Bloch, 1963).

Mycoplasma laidlawii provides another example. The composition of these organisms can be changed by varying the growth medium. Membranes with different degrees of saturation in the fatty acids of the membrane

lipids are obtained. In all cases a transition is observed in the membrane which corresponds to the transition temperature of the extracted lipids dispersed in water (Steim et al., 1969b). Homeoviscous adaptation, the ability to maintain a constant membrane fluidity at the temperatures of growth (Sinensky, 1974) is also found in E. coli (Sinensky, 1974), Bacillus stearothermophilis (McElhaney and Souza, 1976) and in the synaptosomal membranes of acclimated goldfish (Cossins, 1977). In the last study cited, increased membrane fluidity of cold-acclimated goldfish was correlated with a decrease in the proportion of saturated fatty acids of the major phospholipid classes.

The functional consequences of the homeoviscous responses relate to all processes which are dependent on membrane viscosity. These include compensations in the passive permeability of the membrane to ions and other metabolites (see e.g. Papahadjopoulos, 1973) as well as the activity and kinetics of membrane-bound enzymes (see e.g. Swan, 1974 pp. 171-175) among others. We might notice in passing, that the discussion applies to most biological phospholipid systems which are bilayer membranes. It is justified, therefore, to question what functional consequences of changing temperature effect would occur to a phospholipid system like lung surfactant which comprises lipids in both bilayers and monolayers and it is the latter form which is functional.

6. Physical State of Lipid and Surfactant Function

It was pointed out by Clements and Tierney (1965) that the ability of a surfactant film to produce low surface tension is dependent on both equilibrium and kinetic properties (i.e. reaching low surface tension and stable in a highly compressed state), and that these are in turn affected by the compositions of the film and aqueous phase, and by

temperature.

There were a number of studies to investigate the effect of temperature on the surface tension of lung surfactant. These in vitro procedures usually involved measuring the surface tension of lung lavage fluid or saline extracts from homogenates of mammalian lung and results were reported in terms of pressure-volume or pressure-area curves. It was found that temperature changes within the range 21-38°C did not influence the relationship between surface tension and area of human lung extracts (Avery and Mead, 1959). However, Clements and Trahan (1963) observed that heat diminished inflation pressures and raised deflation pressures as rat lungs were filled with gas. Also at functional residual capacity (40% of maximum lung volume), there was a drop in pressure as temperature increased. These data suggested that heating increased minimum surface tension and decreased maximum surface tension. After DPPC was identified as a major constituent of lung surfactant in mammals (Brown, 1964), the effect of ambient temperature in the range 15-29°C was investigated on the racemic form of synthetic lecithin. No significant changes were observed (Kuenzig et al., 1965). In contrast, Tierney and Johnson (1965) found that an increase in temperature accelerated the rate by which surface tension reached equilibrium. They also observed that some lung extracts when studied at 31°C did not develop surface tension less than 10 mN/m during the 5 minute compression-expansion cycles. Upon cooling the lower surface tension reappeared. In discussing the results of monolayer studies with regard to acyl chain length and temperature effect, Phillips and Chapman (1968) first suggested that lipid phase transition might be important for alveolar

surfactant to function. This suggestion rested on the observations that synthetic DPPC in aqueous dispersion shows a thermal phase transition at 41°C , from a condensed to a liquid expanded state. X-ray studies indicated that this transition is accompanied by an increase of 30% in bialyer area (Chapman et al., 1967). Since the temperature in a mammalian lung is lower than the critical transition temperature, the DPPC film will experience a two dimensional condensation unless constrained from doing so. They contended that this transition may be of significance in the continuous compression and expansion loop that lung surfactant undergoes during respiration. Then, it was found that lung surfactant exhibited a thermotropic phase transition (Steim et al., 1969a). They also reported that the surface properties of surfactant were temperature dependent and could be correlated with phase transition temperature. Thermal effect on lung surfactant was again studied by Lampert and Macklem (1971) who measured surface tension of lung extracts and saline washings from $15-40^{\circ}\text{C}$. They observed that minimum surface tension in compression was increased by a rise in temperature whereas maximum surface tension on expansion was decreased, confirming the findings by Clements (1967).

It was observed by Träuble and coworkers (1974) that while the possibility of a phase transition of lung surfactant directly affecting lung mechanics exists, a thermal phase transition cannot be responsible in homeothermic mammals. Instead, they reasoned that a phase transition could be induced by pressure. Their detailed in vitro measurements led them to propose that at 37°C the mammalian lung surfactant is at a critical state such that small changes in pressure would induce spontaneous opening and closing of the alveoli.

In view of the information we have just referred to, it appears that there is considerable evidence suggesting that the physical state, of lung surfactant, which consists mainly of phospholipids could be important for function. If this is the case, one would expect homeoviscous adaptation to take place in lipids of lung surfactant of air-breathing ectotherms acclimated to different temperatures.

Although preliminary measurements had been made on frogs, they were not used in this study because of the possible complication of cutaneous respiration in these animals, map turtles, Malaclemys geographica, were chosen because they are not skin-breathers and for practical considerations such as availability and ease of maintenance.

7. Respiratory Physiology of Turtles

It is perhaps unfortunate that so little is known about the respiratory physiology of Malaclemys geographica, and in fact, of reptiles in general. The discussion that follows applies to reptiles in general and whenever studies on turtles closely related to map turtles, Malaclemys geographica, are available they would be referred to in greater detail.

(a) Lung morphology: If we take an evolutionist's view, we will find in the reptilian lung there is the beginning of a separation of airways, or bronchii from the alveoli, which reaches its peak in mammals (Krogh, 1941). The lungs in many species are quite heterogeneous and the degree of compartmentalisation is directly proportional to the resting and active oxygen uptake. The maximum lung volume is quite large in comparison with that of mammals whereas the surface area is only a hundredth as great (see e.g. Wood and Lenfant, 1976). The pulmonary surface area of reptiles is approximately 1% of a mammal of equal size.

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This difference is quite consistent with the approximately 10-100 fold difference in oxygen requirements of reptiles and mammals (Brett, 1972). Several considerations reveal that reptilian lungs are appropriately developed: The lung of Varanid lizards is subdivided extensively into alveoli throughout the entire lung to meet the high oxygen uptake (Brett, 1972). Unlike in mammals where maintenance of a constant ratio of oxygen uptake to lung surface area is provided by a variable number of alveoli per unit lung volume and, as a result, small mammals tend to have small alveoli (Tenney and Remmers, 1963), the alveolar size and number per unit lung volume are constant in reptiles. Increased surface area arises from increasing the ratio of alveolar to hollow lung area (Tenney and Tenney, 1970).

(b) Mechanics and patterns of ventilation: Mammals are known to ventilate by subatmospheric-pressure breathing. This is accomplished by actively enlarging the thorax during inspiration by lowering the diaphragm and/or orienting the ribs. The pressure in the pleural cavity and alveoli is lowered to subatmospheric and, consequently, air flows in. The thorax and lung then recoil positively to their resting position along with the surface forces of the alveoli at work to push air out and expiration is accomplished (Comroe, 1965). In spite of the absence of a diaphragm it is now clear that reptiles utilize this same mode of breathing, (see e.g. Wood and Lenfant, 1976). In snapping turtles, Chelydra serpentina, which belong to the same order as map turtles, Malaclemys geographica, four major groups of muscles are capable of varying the volume of visceral cavity to provide ventilation when it is on land (Gaunt and Gans, 1969). A pattern analogous to changes in mammalian thorax is produced so that inspiration is in response to subatmospheric pressures

and expiration occurs on relaxation of the muscles. While submerged, the animals take advantage of hydrostatic pressure to squeeze the visceral cavity on expiration (Gaunt and Gans, 1969).

(c) Gas exchange: Keratinisation of the skin in reptiles dictates that most gas exchange has to be pulmonary. However, in aquatic species, an extrapulmonary component of gas exchange through the skin, buccopharynx or cloaca also exists (Wood and Lenfant, 1976). Because of its relatively high diffusivity (20 times that of oxygen), carbon dioxide is readily eliminated by diffusion through moist skin, even in air-breathing mammals. Thus the cutaneous loss of carbon dioxide as a percentage of pulmonary elimination is 85% in a lizard; 7.6% in a rabbit and 1% in man (Prosser, 1961). The data obtained for the elimination of carbon dioxide through the skin indicate great variability in aquatic species ranging from 33% of total carbon dioxide output in elephant trunk snake, Acrochordus javanicus, to 94% in the yellow bellied sea snake, Pelamis platurus (Graham, 1974; Standaert and Johansen, 1974). It must be noted that these are all shell-less marine snakes. Only 4% of total carbon dioxide output is disposed of through skin in a terrestrial lizard, Sauromalus obesus, during normal ventilation (Crawford and Schultetus, 1970). Data for turtles are not available.

Because of its relatively low solubility, oxygen diffuses much more slowly than carbon dioxide through keratinized skin, and aquatic oxygen uptake is always less than aquatic carbon dioxide elimination. Studies with musk turtle, Sternotherus odoratus, which belongs to the same order as map turtles, Pseudemys SPP and Pseudemys scripta, indicate a very minor contribution of aquatic oxygen uptake to the total respiratory requirement (see e.g. Wood and Lenfant, 1976). In contrast, aquatic

elephant trunk snake, Acrochordus javanicus, obtains up to 8% of the total oxygen uptake through skin diffusion (Standaert and Johansen, 1974).

It is recognized that the effect of temperature would induce some changes in a number of parameters of respiratory physiology of ectotherms, or turtles specifically, so that the acid-base balance can be regulated (see, e.g. Howell and Rahn, 1976). Here are some of the temperature dependent variables: (a) arterial pH which varies inversely with temperature (b) arterial carbon dioxide which increases with temperature (c) production of carbon dioxide which increases with temperature. The relationship of some of these changes to the findings of our study shall be referred to in greater depth in the Discussion section.

(d) Lung surfactant: Information on this particular area is at best fragmentary and the conclusions of a number of reports can only be tentative. Miller and Bondurant (1961) by measuring the surface tension lowering capacity of saline extracts from lungs of amphibian, reptile, bird and mammal deduced that the presence of this distinctive surface active material was limited to mammalian lungs. Other investigators later reported that lungs of snakes and crocodiles did not have "normal" surface activity compared to mammals (Avery and Said, 1965; Clements, 1962).

The surface tension lowering effect can also be measured by observation of bubble stability (Pattle, 1958). Bubbles from saline lung extracts are viewed under a microscope, the diameter of a particular bubble is noted when it is first squeezed into distilled water and then at different time intervals. The stability is indicated by the ratio of the original diameter and the contracted diameter. Then there is the

phenomenon called "clicking" which is the alternate flattening and returning to spherical shape of a bubble from lung foam when it is placed below a glass slip in de-aerated water. This also serves as an index of stability of the bubbles (Pattle, 1965). Bubbles squeezed from the lung of slow worm, Anguis fragilis, a legless lizard, are normal compared to bubbles of mammalian lung, but there are few of these stable bubbles and after collapse and reinflation of the lung, the bubbles are not stable (Pattle and Hopkinson, 1963). Since the "clicking" behaviour is somewhat different from that of bubbles from mammalian lungs, the authors concluded that reptile lungs have a lining film not identical with that of bird or mammal, but still capable of lowering surface tension to a low value, and the reserve of this lining film is very small. It is noticed in the afore-mentioned reports, that the temperature at which the reptiles were kept and the assay temperature were not specified.

A rather detailed analysis of lipid composition of lung from several species was carried out by Harlan et al. (1966). Turtle lung was found to contain 48 mg of phospholipid per g of dry lung tissue. Lecithin made up 47-50% of total phospholipid and palmitate was present in high concentration. Though the phospholipid concentration in mammals was considerably higher, they found no significant differences between the species with respect to phospholipid and fatty acid residues. The results were interpreted to be consistent with previous findings that greater amounts of pulmonary phospholipid are present in mammals than in species like turtles whose lung washings show low surface activity. However, the authors conceded that "a direct correlation between the total phospholipid content of lung tissue and surface tension properties of these lungs cannot be drawn". In an attempt to relate the importance of lung

surfactant to the evolutionary development of lung in different species, Clements and coworkers (1970) did find surfactant in turtle as well as in other air-breathing species. The amount correlates well with their respective alveolar surface area. The data also suggested to them that pulmonary surfactants of non-mammalian vertebrates are probably also rich in saturated PC. Evidence was shown that there are two types of cell in the lung of the rat snake, Elaphe obsoleta obsoleta, resembling type I and type II cells of mammalian lung (Brooks, 1970). Cells of the second type contain entities similar to lamellar bodies in mammalian lungs, but their internal configuration is not clear. Lamellar bodies have also been reported in lung epithelial cells of snakes and lizards (Okada et al., 1962).

It can be seen from this brief summary of the available information that lung surfactant is present in turtles, as in other air-breathing vertebrates, and saturated lecithin may be responsible for the lowering of surface tension by forming a monolayer at the alveolar air-water interface, like its mammalian counterpart. However, at temperatures lower than 37°C other lecithins may also be able to lower surface tension effectively. The confusion over the existence of such an alveolar lining in ectothermic air-breathers could have arisen from a lack of correlation between the environmental temperature of the animals and the temperature at which surface activity of the lung lavage fluid or lung extracts were carried out, as the results of the present study would indicate.

8. Objectives and Approach of the Study

The concept that homeoviscous adaptation occurs in biological phospholipid systems like cell membranes serves as the starting point of

our work. There are indications that microviscosity of phospholipids in lung surfactant is important for the well-known physiological function of lowering of surface tension in the alveoli. In vitro evidence suggesting the possible, crucial relationship between lipid phase(s) and surface properties, and hence the proper functioning of lung surfactant has also been shown. The experiments in this thesis were designed to investigate the effect of temperature on the lipid composition of lung surfactant in map turtles which were acclimated to a wide range of temperatures from 5-32°C. Detailed lipid analyses were performed on saline lavage fluid obtained from the lung of the acclimated animals. Temperature induced changes in lavage fluid were compared to lung tissue with respect to phospholipid content, proportion of phospholipids, relative amounts of fatty acid residues in each lipid class and the saturated lecithin molecular species. The results were evaluated in the light of possible homeoviscous adaptation in lung surfactant system, while other mechanisms were also considered. The objects of this study were to gain some insight into the significance of physical state of phospholipid in lung surfactant using an in vivo experimental system analagous to the bacterial systems used to investigate homeoviscous adaption in membranes.

Materials and Methods

1. Materials

Unless otherwise noted, all chemicals and solvents used in this study were reagent grade products obtained from Fisher Scientific Company or Canadian Laboratory Supplies, Dartmouth, Nova Scotia and reagent grade methanol and chloroform were double distilled.

99.9 mole % methanol, 99 mole % chloroform, hexanes (pesticide grade) and carbon disulphide (spectro analytic grade) were purchased from Fisher Scientific Company, Dartmouth, Nova Scotia. Sodium pentobarbital was obtained from M.T.C. Pharmaceuticals Limited, Hamilton, Ontario. Phospholipid standards were products of Serdary Laboratories, London, Ontario. Silica gels G and HR were purchased through Brinkmann Instruments Limited, Toronto, Ontario and were products of E. Merk AG Darmstadt, Germany. 10% EGSS-(ethylene glycol succinate silicone, a polyester-methyl silicone polymer) on gas-chrom P 120 merk, and reference mixtures of fatty acid methyl esters were obtained from Applied Science Laboratories, State College, Pennsylvania. 99.9% indium standard was purchased from Perkin-Elmer, Montreal, Quebec. 99 mole % hexadecane was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Dipalmitoyl-phosphatidyl-choline standard was acquired from Sigma Chemical Company, St. Louis, Missouri. Dioleoyl phosphatidyl-choline was synthesized from egg yoke lecithin in our laboratory. Neutral aluminum oxide (Woelm Alumina, activity grade I) was obtained from ICN Pharmaceuticals Company, Ohio.

2. Glassware and Surgical Instrument Cleaning

All glassware and chromatograph plates were soaked overnight in

chromerge solution (Monostat, New York). They were rinsed six times with tap water, six times with distilled water and oven-dried before use. Pipettes were soaked at least overnight in chromerge solution (Nalgene Laboratory Company, New York). They were rinsed in a pipette washer for one day, allowed to soak in distilled water overnight and then dried.

After use, surgical instruments were soaked in soapy water for a short while and rinsed thoroughly with tap water and distilled water. They were then placed in methanol for 30 minutes and allowed to air dry. The syringe and blunted needle for lavage was rinsed at least 20 times with distilled water before use.

3. Animals and Food

Map turtles, Malaclemys geographica, were supplied by Arch Reid Enterprise Limited, Ontario. Both male and female turtles were used. Most of them measured about 15 cm in carapace length; a few of them measured about 25 cm. They were held in a tank of running water at ambient temperature for 1-16 weeks before being placed in the fixed temperature tanks. Minced beef, purchased from a local supermarket, in which 5 ml of vitamin supplements (Infantol) were mixed, was used as feed during the holding and acclimation periods. Attempts to feed the turtles with raw fish and mealworms were unsuccessful and were abandoned.

4. Acclimation of Turtles

The animals were acclimated to four temperatures; 32 ± 1 , 22 ± 1.5 , 14 ± 1 and $5 \pm 1.5^{\circ}\text{C}$ for at least one month. The 32°C tank consisted of an Aquarium Instant Ocean System 30" x 30" x 14", 50 gallon capacity, (Model CS50), into which 8-10 turtles were kept at a time. A platform

of a cement slab on bricks was constructed inside the tank and it was water-filled to the level of the platform. Water was aerated and the top was covered with glass to allow better temperature control. Temperature was regulated by means of a heating unit and a compressor.

A translucent plastic tray measuring 36" x 22" x 10" containing some bricks and water to the level of the bricks served as the 22°C chamber for 3-6 turtles. Temperature was regulated by regulating room temperature.

A second Instant Ocean Aquarium (13" x 30" x 14", 25 gal) with a platform of bricks served as the 14°C tank. Six turtles were kept there.

A 5°C cold room was used to house a long rectangular tank measuring 30" x 16" x 14". It contained ten turtles and tap water enough to cover a number of scattered bricks.

The turtles were fed twice a week. Water was changed after feeding. Animals at 5 and 14°C ate very little or not at all. Turtles which showed morphological abnormalities were rejected.

5. Lung Lavage and Tissue Preparation

(a) Euthanasia: The first few turtles were beheaded. This method was unsatisfactory in that very often blood was found inside the lung and the lavage had to be discarded. The method eventually adopted was to inject 3 ml of sodium pentobarbital into the body cavity of the turtle via the soft region between the hind limb and tail. The animal was maintained at its appropriate environmental temperature until it died (about 10-15 minutes).

(b) Lung lavage: The plastron was opened with a rongeur and the

heart and liver, which lay on top of the lung, were removed. Without further freeing the lung from other membranous attachments, lung lavage was carried out in situ. A blunted needle head was inserted into the trachea, tied firmly in place and then connected to a 5 ml syringe. The set up was shown in Fig. 4. Physiological saline 0.72% (w/v) (calculated according to Dawson et al, 1969 pp. 508) was slowly pumped into and withdrawn from one lobe of the lung six times. Approximately 90% of the volume of saline solution passed into the lungs was recoverable. Each lobe was washed separately with three successive 5 ml portions of saline (for 25 cm turtles 10 ml portions were used). The washings and organs for subsequent processing were chilled on ice and the former were centrifuged for 5 min. at 500 g (in an SS34 rotor in a Sorvall RC2B centrifuge). The supernatant was examined using phase contrast microscopy. The number of cells were counted to ensure the phospholipid obtained for analyses could not have come from cells sloughed out during lavage. Presumably some of the surfactant lipids would be lost as they were deposited with the cells during centrifuge.

(c) Treatment with the lung: After the lavage, the lung was cleared of the exterior membranes. The trachea and bronchii were removed. This usually took about 10 minutes and was carried out on ice. The lung was squeezed to exclude the saline left behind during lavage and the surface was blotted dry. The fresh weight was obtained using a Sartorius balance.



Figure 4: Demonstration of lung lavage.

6. Lipid Extraction

The procedure followed was based on the method of Bligh and Dyer (1959).

(a) Lung surfactant: The supernatant was shaken about 200 times in a stoppered graduated cylinder with 99.9 mole % methanol and 99 mole % chloroform, so that the ratio of chloroform/methanol/saline was 1:1:0.9 (v/v/v). The volume of the chloroform phase was noted and the upper phase of saline-methanol solution was removed by aspiration. The lipid extract in chloroform was stored at -25°C until further processed.

(b) Lung: The weighed lung was cut into small pieces and transferred to a test tube (22 mm x 175 mm). By assuming water constitutes 90% of the wet weight of the lung, the volume of saline required to make up the total amount of water with the composition: chloroform/methanol/saline in the ratio of 4:8:3.2 (v/v/v), was calculated. The lung was homogenized for 2 minutes using a Polytron (Brinkmann Instruments, PT10) at maximum rate, while the content was continually chilled by ice surrounding the test tube. Chloroform was added to the mixture, so that the composition was chloroform/methanol/saline in the ratio of 8:8:3.2 (v/v/v) and homogenization was continued for 30 seconds. The final composition of the mixture was adjusted by adding saline to a ratio of 8:8:7.2 (v/v/v) with respect to chloroform/methanol/saline. It was homogenized for another 30 second period. For extraction, the chloroform used was 99 mole % and methanol 99.9 mole %. The mixture was vacuum filtered through a Buchner funnel (9 cm in diameter) using Whatman No. 4 filter paper which gave good retention and a short filtration time. The filtrate was transferred to a measuring cylinder and

the volume of the chloroform fraction was recorded. The recovery of chloroform was approximately 75%. The methanol saline phase was aspirated away. A few grains of hydroquinone were added to the lung lipid extract before storage at -25°C in small glass vials with aluminum lined caps.

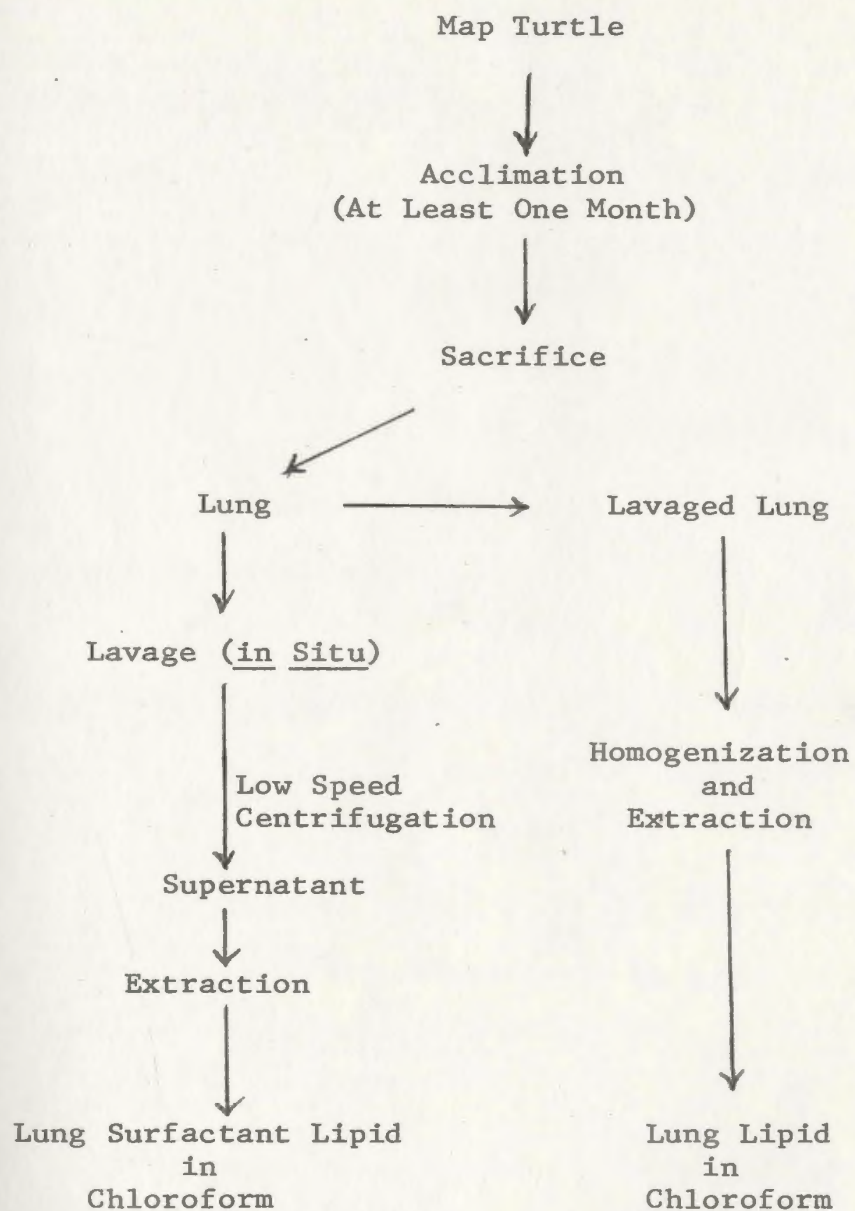
No gross morphological differences were observed between the lungs of animals acclimated to various temperatures. In summary, the protocol can be represented as the next page.

7. Phospholipid Analysis

The procedures employed in this thin layer chromatography technique were small modifications of those given by Skipski and Barclay (1969).

(a) Pouring of plates: Five chromic acid washed glass plates and two end plates were laid on a spreading template (Desaga, Heidelberg) and were further cleaned by tissue paper soaked with chloroform/methanol 1:1 (v/v). 35 g of silica gel G type 60 was stirred with 78 ml of distilled water in a beaker. The slurry was quickly transferred to an applicator (Desaga, Heidelberg) and a coating of 0.25 mm thick was spread uniformly over the plates, which were gently separated and allowed to air-dry.

(b) Sample application and separation: Immediately before use, the plates were activated at 110°C for 45 minutes and cooled to room



The protocol of experimental procedures for the isolation of lipids.

temperature in a desiccator. Material for phospholipid analysis was taken to dryness under N_2 in a conical tube. The residue was dissolved in 25 μ l of 99 mole % chloroform and applied carefully to the ruled chromatogram by means of a 25 μ l syringe (Hamilton, Canadian Laboratories). The developing tank, lined with filter paper, was saturated with the solvent for one hour before chromatography. A two dimensional separation was used and the chromatograms were first developed in the solvent system 65:30:5 chloroform/methanol/ammonia (v/v/v). The chromatograms were developed until the solvent front was within 3 cm from the top. They were then taken out and vacuum dried for 30 min. A second dimension separation was achieved by using another solvent system 80:25:6:2 chloroform/methanol/acetic acid/water (v/v/v/v).

To get rid of acetic acid, the chromatograms, after development, were vacuum dried for 45 min. before detection and identification. Normally, five chromatograms were being processed simultaneously and the total volume of solvent required for development was approximately 110 ml.

(c) Identification and detection of phospholipids on chromatogram:

The relative positions of the standard phospholipids on G gel plates were obtained by one dimensional separation in the two solvent systems. The mixed standard phospholipid mixture were applied along with the sample in two dimensional separation. The first few chromatograms were sprayed with different specific reagents to further confirm the identity of particular phospholipids. PE and PS were identified by reaction with ninhydrin. Periodate-Schiff spray was used to identify PI and PG. PI appeared first in a dark green colour and PG gradually showed

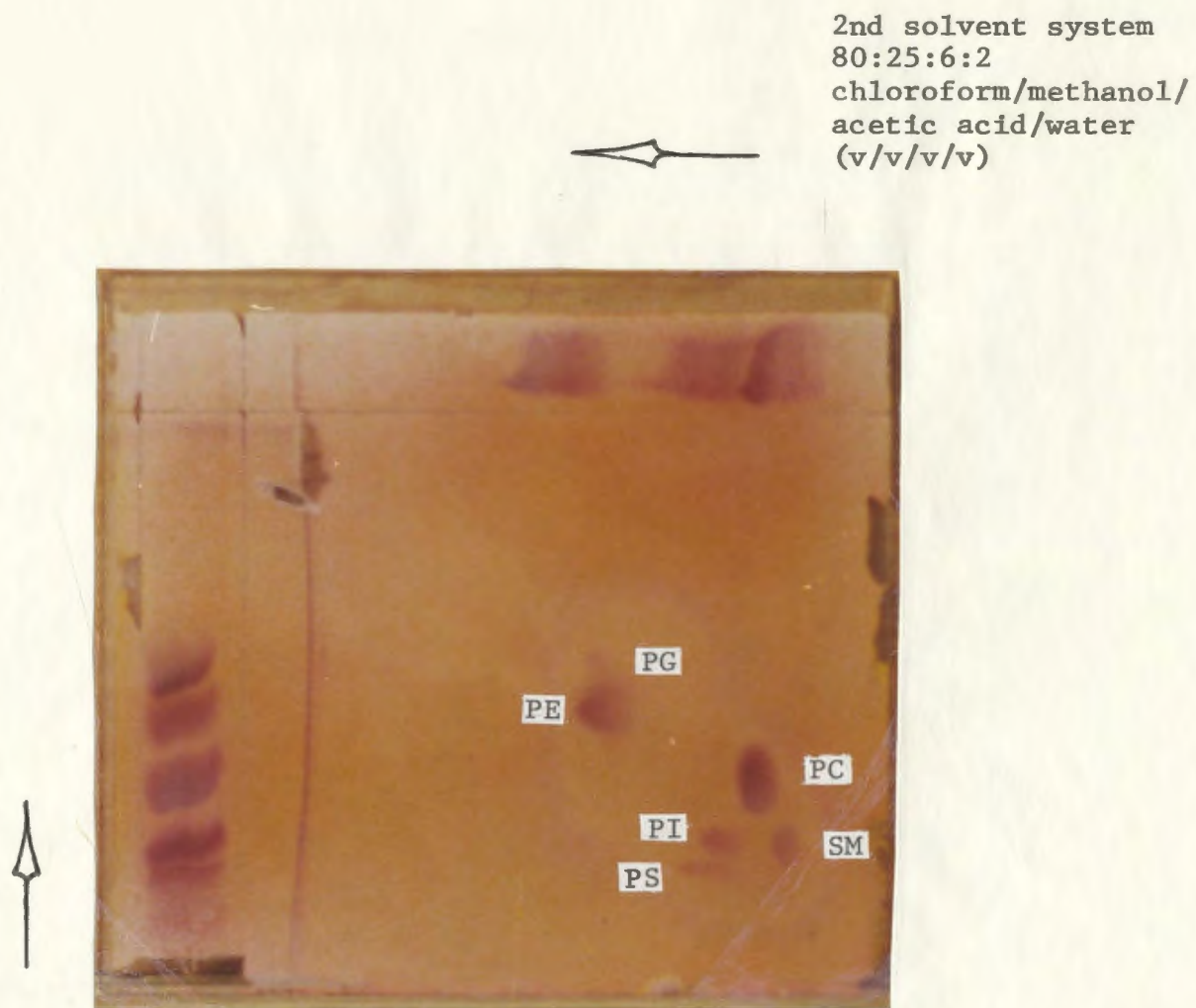
up in light purple. SM spot was faintly dark (Kates, 1972 pp. 437-438). PC which ran quite distinctly apart, was readily recognized. The pattern of phospholipids after development appeared as in Fig. 5. The conditions leading to this separation were arrived at using lipid extracts of frog lungs. Frogs were subsequently abandoned as the experimental animal for a number of reasons. A re-examination of periodate-Schiff reactivity in turtle lung preparation indicated some overlap in PE and PG spots. Since the total phosphorus in this region was small in turtle lung surfactant preparations, further attempts to achieve better separation were not made. It was found that PG was absent in turtle lung surfactant by using periodate-Schiff spray.

In subsequent experiments the routine detection of phospholipids involved putting the chromatogram into a tank saturated with iodine vapour. The lipid spots appeared yellowish brown. This method was used for all purposes except when the phospholipids were to be recovered for fatty acid analysis. In this case, dichlorofluorescein spray (0.1% of the dye w/v in 95% ethanol) was employed. Fluorescent spots of lipid were detected when viewed under ultra-violet light.

8. Fatty Acid Analysis

The analysis was based on the technique of gas-liquid chromatography, first introduced by James and Martin (1952). The principle involves partitioning of the components of the mixture in the vapour state between a mobile stable gas phase and a stationary non-volatile liquid phase on an inert support.

Materials for fatty acid analysis was taken to dryness under N_2 . The residue or gel containing phospholipids was dissolved in 2 ml of 6% (v/v) sulphuric acid in 99.9 mole % methanol to which a small amount



1st solvent system
65:30:5 chloroform/methanol/ammonia (v/v/v)

Figure 5: Two-dimensional separation of lipids after prolonged staining with periodate-Schiff reagent.

of hydroquinone had been added. Transesterification of fatty acids was carried out in sealed tubes at 85°C overnight. Following transmethylation the tubes were cooled and opened. 1.5 ml of hexanes was added and vortex-stirred. The hexane phase was aspirated and transferred to another test tube. The methanol phase was washed twice more with hexanes. The hexane extract(s) were pooled and washed with 1 ml of water. In a tapered centrifuge tube, the extracts were evaporated to dryness under N₂ and redissolved in a small volume of carbon disulphide (25-50 µl).

Fatty acid methyl esters were analysed using a Packard (Model 7400) gas chromatography. The flame ionization detector assembly and the injection port were maintained at 230°C. A 6-foot glass column with an internal diameter of .2 mm was packed with 10% ethylene glycol-succinate-methyl-silosane copolymer on gas chrom P as the stationary phase. The moving phase, high purity N₂ gas, flowed at 40 ml/min. at an inlet pressure of 40 psig. The ionization flame was supplied with H₂ at 35 ml/min. at 30 psig, and air at 250 ml/min. at 20 psig. The column was operated isothermally at 160°C or 190°C.

Individual fatty acids were identified by comparing the retention times of fatty acid peaks in the sample with those of reference compounds. The peaks in the chromatograph were integrated by planimeter (Hruden, Canadian Scientific Products).

9. Isolation of Saturated Phosphatidylcholine

The procedures employed were based on the method of Henderson and Clayton (1976). The method makes use of the fact that fully saturated phospholipids precipitate out of solution in organic solvents at low

temperature to a greater extent than do phospholipids containing unsaturated fatty acids. It has been shown that for saturated phospholipids, the longer the fatty acid chain, the higher the temperature at which the phosphoglyceride remains at the origin of a thin layer gel plate when developed in a proper solvent system. The nature of the alcohol groups also affects the separation. Given the same fatty acid composition, phosphatidylcholine and phosphatidyl glycerol migrate at temperatures at which phosphatidyl ethanolamine remains at the origin. Phosphoglycerides having any degree of unsaturation fail to remain at the origin, no matter at how low a temperature the chromatogram is developed.

Phosphatidylcholine was isolated from 150-175 μg of surfactant total lipid by the two dimensional technique outlined above. To the phosphatidylcholine-containing gel was added 5 ml of 99 mole % chloroform/99.9 mole % methanol (1:1, v/v) and was shaken vigorously in a tapered centrifuge tube fitted with a glass stopper. The suspension was centrifuged at 85% of maximum speed of the clinical centrifuge (IEC Damon) for 5 minutes. The supernatant was decanted and the gel was washed again first with the same solvent than with 5 ml of methanol alone. The extracts were pooled and taken to dryness under N_2 and redissolved in 50 μl of 99 mole % chloroform.

78 ml of 1.0 mM Na_2CO_3 solution was added to 35 gm of G-HR gels. The slurry was spread on 5 plates with a coating of 0.25 mm. The chromatogram having been activated at 110°C for 45 minutes, the solvent blank and sample phosphatidylcholine were spotted in the separate lanes by 3 successive washings with 40 μl of 99 mole % chloroform. Standards of DPPC and dioleoyl phosphatidylcholine were also applied. The chromatogram was placed in an evacuated desiccator and was allowed to equilibrate in vacuum at -70°C for 1 hour. A solvent system of chloroform/methanol/acetic acid/0.15 M saline 60:30:20:3 (v/v/v/v)

which had been previously equilibrated at -70°C was used for developing the chromatogram. Following development the chromatogram was vacuum dried in a desiccator and allowed to warm up to room temperature. After identification, the saturated, unsaturated PC and the corresponding blank spots were analysed for fatty acid composition with heptadecanoic acid as an internal standard. Unsaturated PC was found to migrate a few centimeters from the origin while saturated PC did not move at all.

10. Phosphorus Determination

Phosphorus was estimated using the following modification of the procedure of Bartlett (1959). The reagents and standard used were as follows:

- (a) Stock phosphate solution, 2.1956 g KH_2PO_4 in 500 ml 1 N H_2SO_4 , was diluted to give 2 μg of inorganic phosphorus per ml.
- (b) 0.25% aminonaphthol sulphonic acid (ANSA) was prepared as follows: 1.0 g of the dry powder was dissolved in 390 ml of 15% sodium metasulphate, and to this was added 10 ml of 20% sodium sulphate, agitated and heated to dissolve. The suspension was chilled and filtered.
- (c) 72% (w/v) perchloric acid.
- (d) 5% ammonium molybdate.

Material for phosphorus determination was taken to dryness under N_2 . The sample was digested in a kjeldahl digestion rack (Aminco) in the presence of 1.0 ml of 72% perchloric acid and some anti-bumping granules. The total volume was made up to 9.0 ml with water. 0.5 ml of molybdate solution was added and vigorously stirred. This was followed by 0.5 ml of ANSA, and after stirring, they were incubated in

a boiling water bath for 10 minutes. The samples were cooled and the optical density was measured at 815 nm using Unicam Sp500 spectrophotometer. The scanning curve for optimum absorption of the standards is shown in Fig. 6.

For gel containing samples, the same procedures were followed except at the end of the 10-minute incubation the gels were separated by centrifugation (IEC Damon) for 5 minutes at speed 6 of a scale of 0-7, and the supernatant was kept for optical density measurement.

2 ml of the diluted phosphate standard was used. Reagent blanks and gel blanks approximating the area of the phospholipid fractions were also included. Whenever possible, each sample was assayed in duplicate.

From the moment when the turtles were sacrificed up to the point when all analyses had been carried out, the entire procedure usually took no more than four days.

11. Isolation of Lung PC

Six turtles were sacrificed and the lung lipid was extracted. PC was isolated by applying 250 mg of the lung lipid to a 6 g aluminum oxide column and was eluted by chloroform methanol solution with increasing methanol in the eluting solvent.

Composition of eluting solvent chloroform : methanol (v/v)	Bed volumes (10 ml)	Fraction
100 : 0	1 x 5 (50 mls)	1
97.5 : 2.5	2 x 2.5 (2 x 25 mls)	2,3
95 : 5.0	2 x 2.5 (2 x 25 mls)	4,5
90 : 10	2 x 2.5 (2 x 25 mls)	6,7
80 : 20	2 x 2.5 (2 x 25 mls)	8,9

The fractions were spotted on a chromatogram and the separation was carried out in the solvent system 60:40:4 chloroform/methanol/water

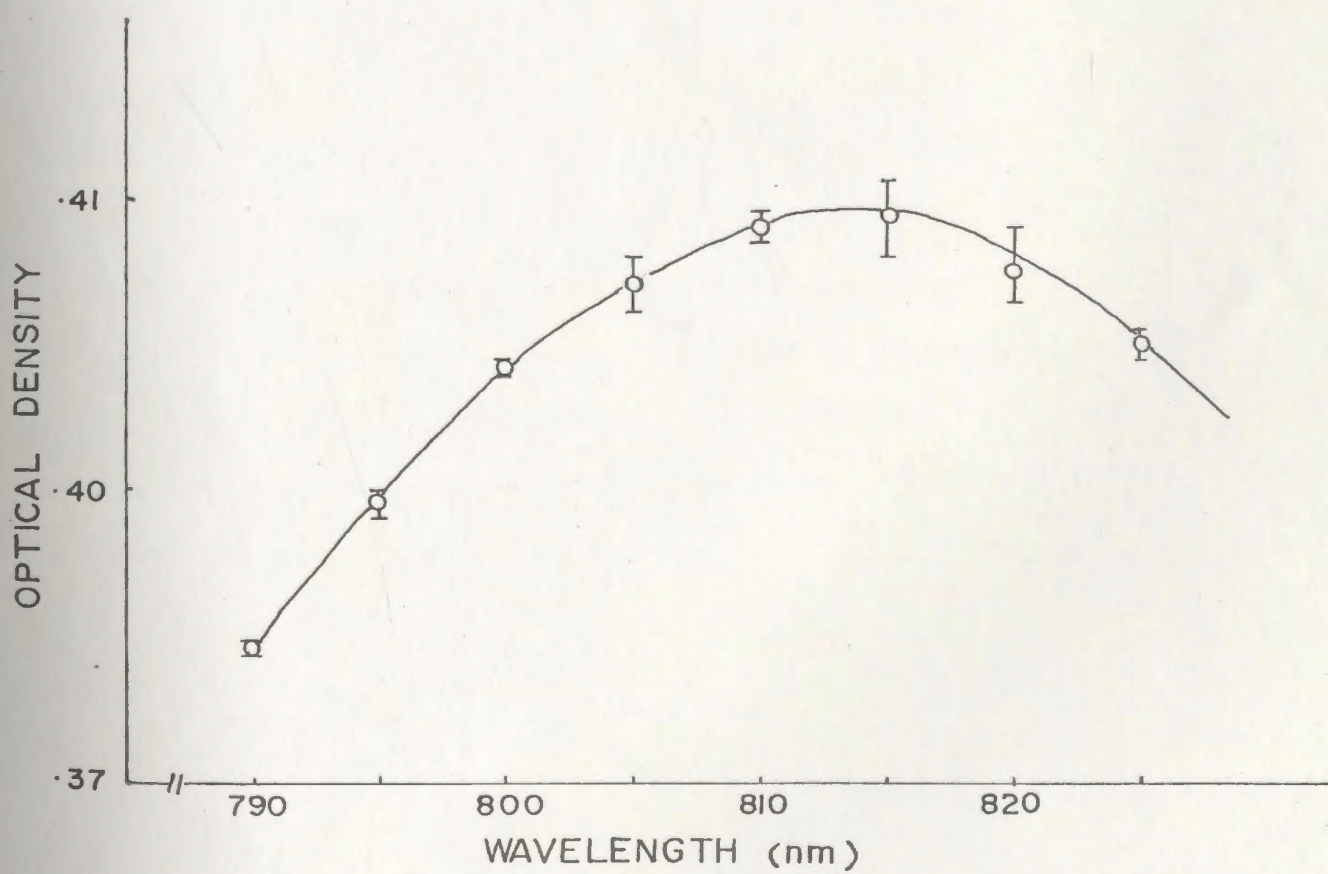


Figure 6: Scanning curve for Bartlett standard. Error bars represent the range.

(v/v/v). It was found that fractions 6,7, and 8 contained most of PC and little of any other lipids.

The fatty acid composition of this lung PC was obtained. By comparing this fatty acid profile with that of surfactant PC at 5°C and 32°C, the amounts of DPPC required to approximate surfactant PC(s) were estimated.

The preparations were subjected to thermal analysis using differential scanning calorimetry.

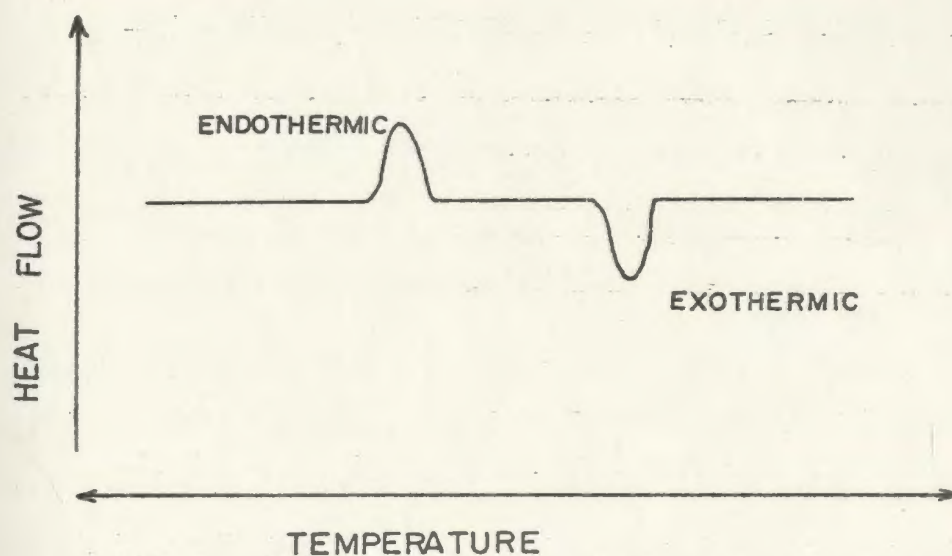
12. Cloacal Temperature Measurement

The body temperature of the turtles was measured by a plastic coated thermister in the cloaca. The thermister was held in place by tying it to the tail with a piece of thread. The turtle was allowed to move freely while the cloacal temperature was read from a digital thermometer (Model 5650, Markson Scientific Incorporation). Temperature readings were recorded every 30 seconds for 5 minutes. The air and water temperature were also recorded using the same instrument.

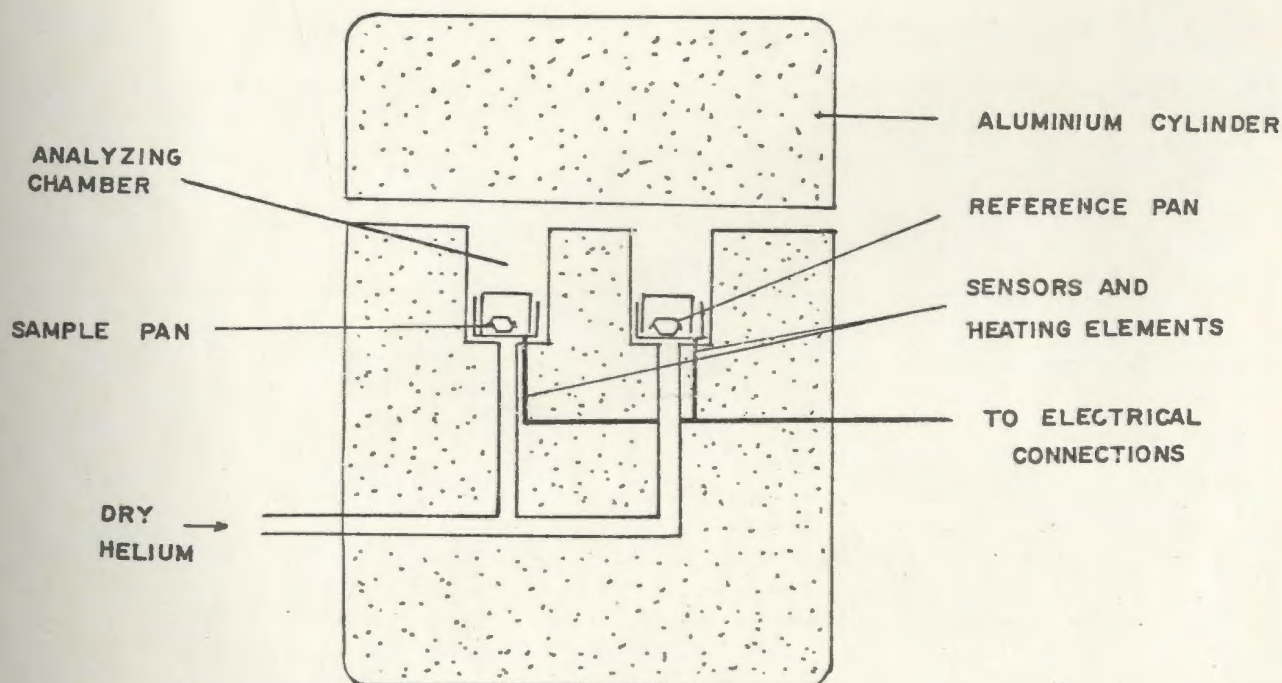
13. Differential Scanning Calorimetry (DSC)

This technique is based on the principle that when a thermally matched sample and reference are maintained at the same temperature throughout heating or cooling, the variation in heat flow in terms of electrical energy to the sample to maintain that temperature during a thermal event can be measured. The temperature at which the event takes place can also be recorded.

In the instrument used (Perkin-Elmer DSC2) the sample and reference are placed in two platinum analysing heads which are enclosed in a large aluminum cylinder (see Fig. 7b). The aluminum cylinder acts to



a. A stylised DSC scan.



b. Diagrammatic representation of the aluminum cylinder and the analysing chamber.

Figure 7: Diagrams to show analysing unit and a stylised scan produced by a Perkin-Elmer DSC2.

maintain a constant ambient temperature, and for our experiment it is immersed in liquid N_2 ($-196^{\circ}C$). The analysing heads are purged with dry helium and the whole is contained within a dry box which is purged with dry N_2 .

The analysing heads are matched platinum containers and each is equipped with a temperature sensor and a heating element. The calorimeter has a control circuit to allow both the sample and reference to be heated or cooled at a constant preselected rate. The energy can be supplied in such a way that both the sample and the reference are always at the same temperature. When a thermal event occurs during programming, it is monitored as the difference in energy to maintain both the sample and the reference at the same temperature. The current difference supplied to the two heads is monitored, amplified and displayed on a strip chart recorder which has an event marker for temperature. A recording of energy supplied vs temperature is obtained. Endothermic or exothermic events are indicated by departures from the baseline (see Fig. 7a). The heat associated with an event can be obtained by integrating the areas under the curves where these events occur. Temperature and enthalpy calibration is achieved using known standards, which in this case were 99.9% indium and 99 mole % hexadecane.

20 mg in 40 μ l of total lipid dispersion was prepared by warming the suspension to $60^{\circ}C$ and vorticing it several times. Approximately 15 μ l of the dispersion was transferred to the sample pan and sealed. Both the sample pan and the air reference pan were placed in the dry box, and dry nitrogen gas was allowed to flow for a few minutes. The

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heads were maintained at 30°C to avoid condensation during the transfer. The analysing chamber was purged with dry helium gas and the gas cooled to 250°K . It was programmed to warm up to a fixed maximum temperature at a selected rate. The sensitivity was adjusted to permit a reasonable recording.

14. Statistical Testing

All results were compared by Student's t-test using computer programmes in the SPSS package (2nd Ed. Nie et al., McGraw Hill Book Company).

Results

1. Acclimation

It was found that the air temperature above the water in each tank was approximately the same as the water temperature, and the cloacal temperature of the turtles was within $\pm 0.1^{\circ}\text{C}$ of the water temperature.

The acclimation period was selected to be at least one month. While the minimum time for acclimation was not known, it was felt that complete adaptation should take place in a month based upon work with fish (see for example, Smith 1976).

2. Phospholipids of Lung and Surfactant at Different Temperatures

(a) Efficacy of saline lavage: To determine the effectiveness of the technique of lavage, phosphorus was measured in successive washings from one lobe of a lung. In this particular case, the lung weighed 8.45 g and was obtained from a turtle kept at 14°C , whose shell length was approximately 25 cm. The results are shown in Fig. 8. The major portion of the lipid phosphorus was recovered in the first fraction of the lavage. The yield of phosphorus was very low in the third and fourth washes. Of the total phosphorus obtainable in 4 washes, 67% was in the first and the first three washes together contained 95%. In all subsequent experiments, three successive washes were pooled for extraction and analysis of lipid. The total yield of these 4 washings was $48.2\text{ }\mu\text{g}$ of P, which compared favorably with $49.7\text{ }\mu\text{g}$ of P in the three washings from the other lobe of the same lung, showing good reproducibility between lobes. It would also appear that the technique of lavage was not causing extensive rupture of epithelial cells, for if the cells were leaking contents such as subcellular organelles and lamellar bodies, it

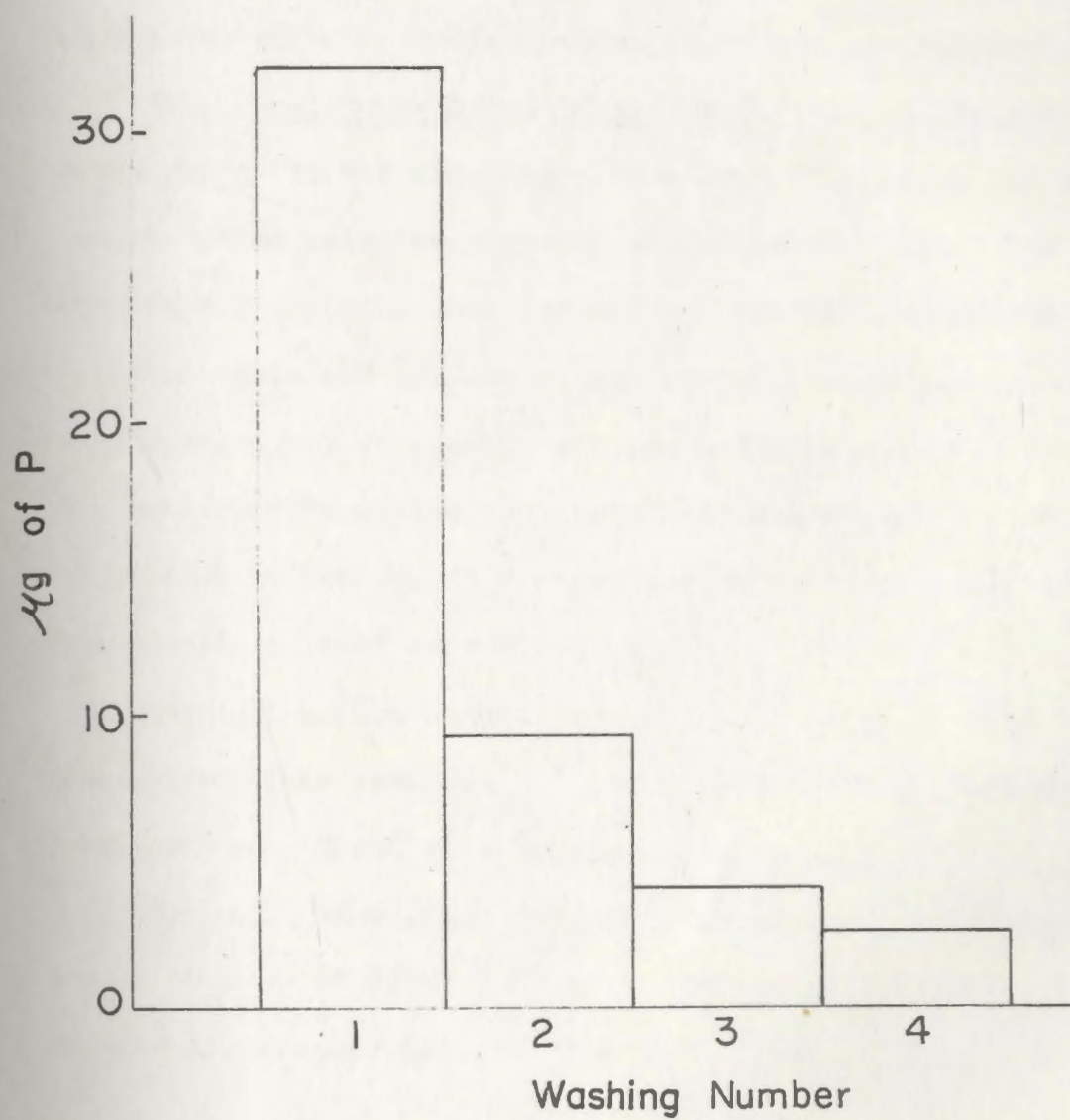


Figure 8: Phospholipid P content in successive washings from one lobe of a turtle lung.

was unlikely that they would be removed by low speed centrifugation, or later be detected under phase contrast microscope, and a decline in lipid phosphorus with successive washes would not be expected (Fig. 8).

(b) Lipid contributed by residual cells in preparation of surfactant: It was imperative to ensure that lipid had not come from sources other than the alveolar lining of the lung. The centrifugation step in the protocol was designed to separate epithelial cells or blood cells sloughed off during lavage from the cell-free surfactant. The supernatant was routinely examined using phase-contrast microscopy. Any cells left behind were counted using a hemocytometer so that an estimation on the possible contribution of these cells to the surfactant lipid could be made as follows:

The cell number ranged from 0-4 in a total of four counts in all the preparations examined. 4 cells were found in this particular sample after a total of 4 hemocytometer counts.

The cells were counted in 25×16 squares of the hemocytometer, each having an area of $1/400 \text{ mm}^2$ and 0.1 mm in depth. The volume of the supernatant counted was $4 \times 25 \times 16 \times \frac{0.1}{400 \times 10^3} \text{ ml}$.

$$\begin{aligned} \text{i.e. cell concentration} &= \frac{4}{4 \times 10^{-4}} \text{ ml}^{-1} \\ &= 10^4 \text{ ml}^{-1} \end{aligned}$$

The diameter of the cells averaged $1/40 \text{ mm}$, assuming the cells to be spherical

$$\begin{aligned} \text{cell size} &= \frac{4}{3} \pi \left(\frac{1}{80} \times 1000 \right)^3 \mu^3 \\ &= 8000 \mu^3 \end{aligned}$$

We had measured the lipid phosphorus in lung which was approximately

200 $\mu\text{g P/g}$ fresh weight. We assumed the cell density to be 1 g per ml.

The lipid phosphorus contributed by the cellular contamination would

$$\text{be } 8000 \times 10^{-2} \times 1 \times 10^4 \times 200 \mu\text{g/ml} = 1.6 \times 10^{-2} \mu\text{g/ml}$$

The same lavage was found to contain 1.91 μg of lipid phosphorus per ml.

Therefore, the phospholipid of surfactant contributed by cellular

$$\text{contamination was approximately: } \frac{1.6 \times 10^{-2}}{1.91} \times 100 \% = 0.83\% = 1\%$$

(c) Lipid phosphorus in lung and in surfactant: In determining the lipid phosphorus of lung and of surfactant per gram of fresh lung tissue, it was found that the weight of the lungs varied considerably between turtles of a similar size. For turtles whose carapace length measured about 15 cm, the lungs weighed around 1.5 g, ranging from 0.74 to 1.78 g. However, no systematic changes in lung weight and gross morphological differences with temperature were observed.

With the exception of a few samples from turtles of 25 cm carapace length, most samples represented the pooling of material from two 15 cm turtles. The results were shown in Table 1.

The lipid phosphorus of surfactant was about 5% of that of lung at 5°C and about 10% at 32°C. The increase was due to the significant increase of phospholipids of surfactant at 32°C, while phospholipids of lung tissue did not show significant changes over the temperature range, a gradual increase of the phospholipids in surfactant with increasing temperature was quite apparent.

3. Phospholipid Composition of Lung and Surfactant at Different Temperatures

The phospholipids were further separated into their classes by thin layer chromatography. During sample analysis, all the individual phospholipids were calculated in μg of P. The percentage

Table 1 - Lipid Phosphorus in Lung and Surfactant from
Turtles kept at Different Temperatures

Environmental Temperature (°C)	No. of Samples	Lipid Phosphorus	
		Lung	Surfactant
5	4	209.0 \pm 82.8	10.7 \pm 2.7
14	4	220.5 \pm 21.5	7.4 \pm 5.0
22	4	184.8 \pm 48.9	13.9 \pm 4.4
32	4	199.3 \pm 23.9	18.6 \pm 1.6

Values are means \pm S.D. μ g of P per gram of lung tissue.

Significant Changes

Lipid	Temperatures	P
Total Surfactant Lipid	32 : 5	0.002
	32 : 14	0.005

Table 2 - Lung Phospholipids of Turtles Kept at Different Temperatures

Environmental Temperature (°C)	Number of Samples	<u>Phospholipids (% by weight)</u>					
		PC	PE	SM	PS	PI	Unidentified
5	4	38.4 \pm 2.4	29.7 \pm 0.8	15.7 \pm 3.2	9.5 \pm 2.4	2.1 \pm 2.4	4.9 \pm 1.2
14	4	43.1 \pm 2.6	27.0 \pm 2.0	15.4 \pm 1.3	8.0 \pm 1.0	3.5 \pm 1.8	3.2 \pm 1.5
22	4	42.5 \pm 2.0	25.5 \pm 1.8	18.1 \pm 2.0	5.5 \pm 2.5	4.1 \pm 0.7	5.7 \pm 0.3
32	4	40.1 \pm 0.9	28.1 \pm 1.8	17.4 \pm 2.7	7.0 \pm 1.9	2.8 \pm 1.1	4.6 \pm 3.1

Values are means \pm S.D. of the percentage of total phospholipid phosphorus recovered in each lipid.

Significant Changes

Phospholipid	Temperatures	P
PC	5 : 14	0.039
	5 : 22	0.038
PE	5 : 14	0.046
	5 : 22	0.006

Table 3 - Surfactant Phospholipids of Turtles Kept at Different Temperatures

Environmental Temperature (°C)	No. of Samples	PC	PE	Phospholipids (% by weight)		PI	Unidentified
				SM	PS		
5	4	70.9 \pm 2.7	5.4 \pm 2.5	3.2 \pm 0.5	2.1 \pm 1.5	9.1 \pm 2.9	9.4 \pm 2.1
14	4	70.7 \pm 3.7	8.4 \pm 3.0	3.9 \pm 0.5	2.7 \pm 1.8	7.6 \pm 1.8	6.8 \pm 4.7
22	4	77.5 \pm 4.8	5.7 \pm 3.8	3.5 \pm 1.0	2.5 \pm 1.4	6.5 \pm 2.0	5.7 \pm 5.8
32	4	77.1 \pm 3.1	6.7 \pm 1.9	3.5 \pm 0.8	2.8 \pm 0.2	10.0 \pm 0.5	1.6 \pm 1.9

Values are means \pm S.D. of percentage of total phospholipid phosphorus recovered in each lipid.

Significant Changes

Phospholipid	Temperatures	P
PC	5 : 32	0.022
	14 : 32	0.037
PI	14 : 32	0.041
	22 : 32	0.043

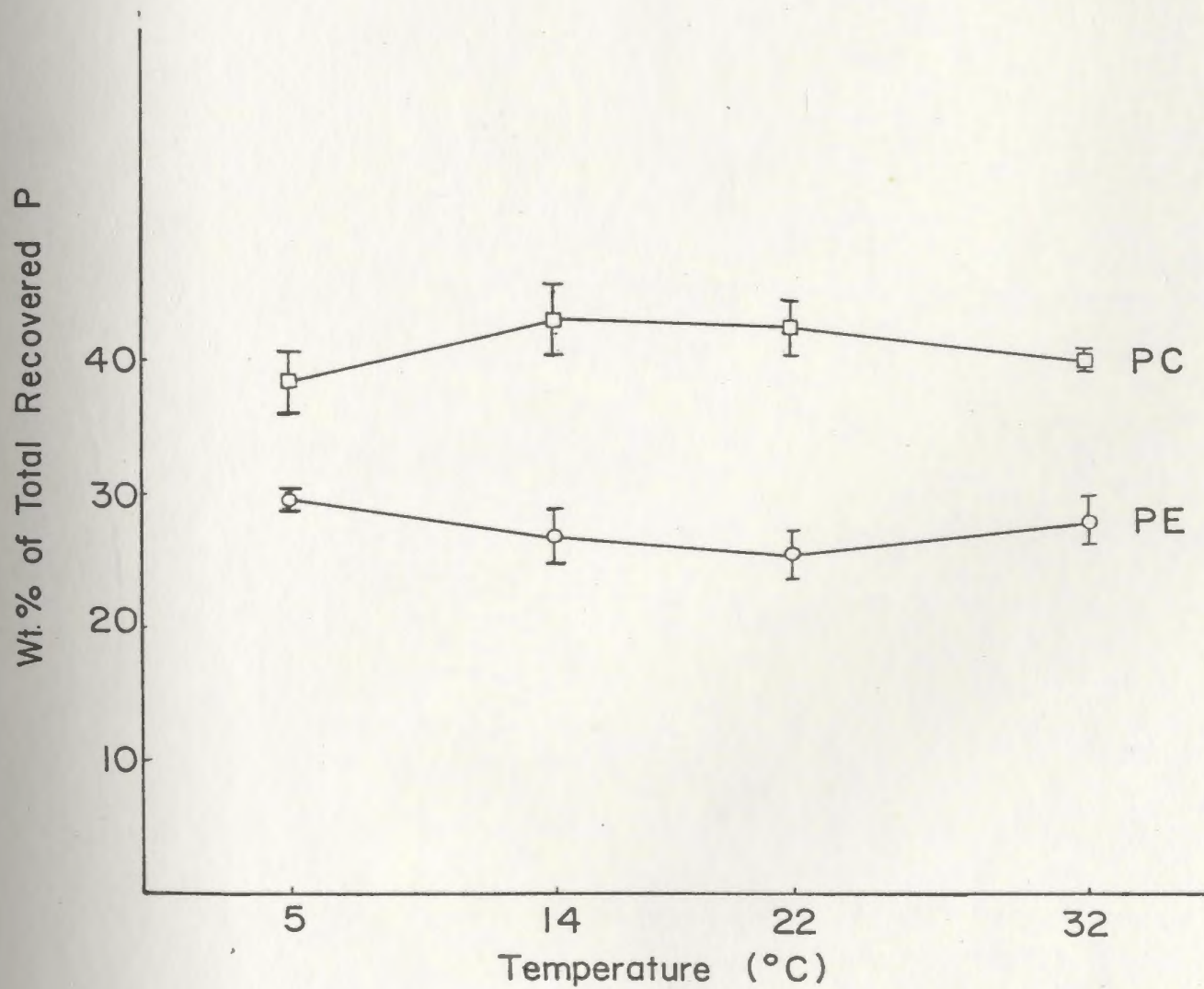


Figure 9: Phospholipids in lung that show significant changes with temperature. Error bars represent S.D.

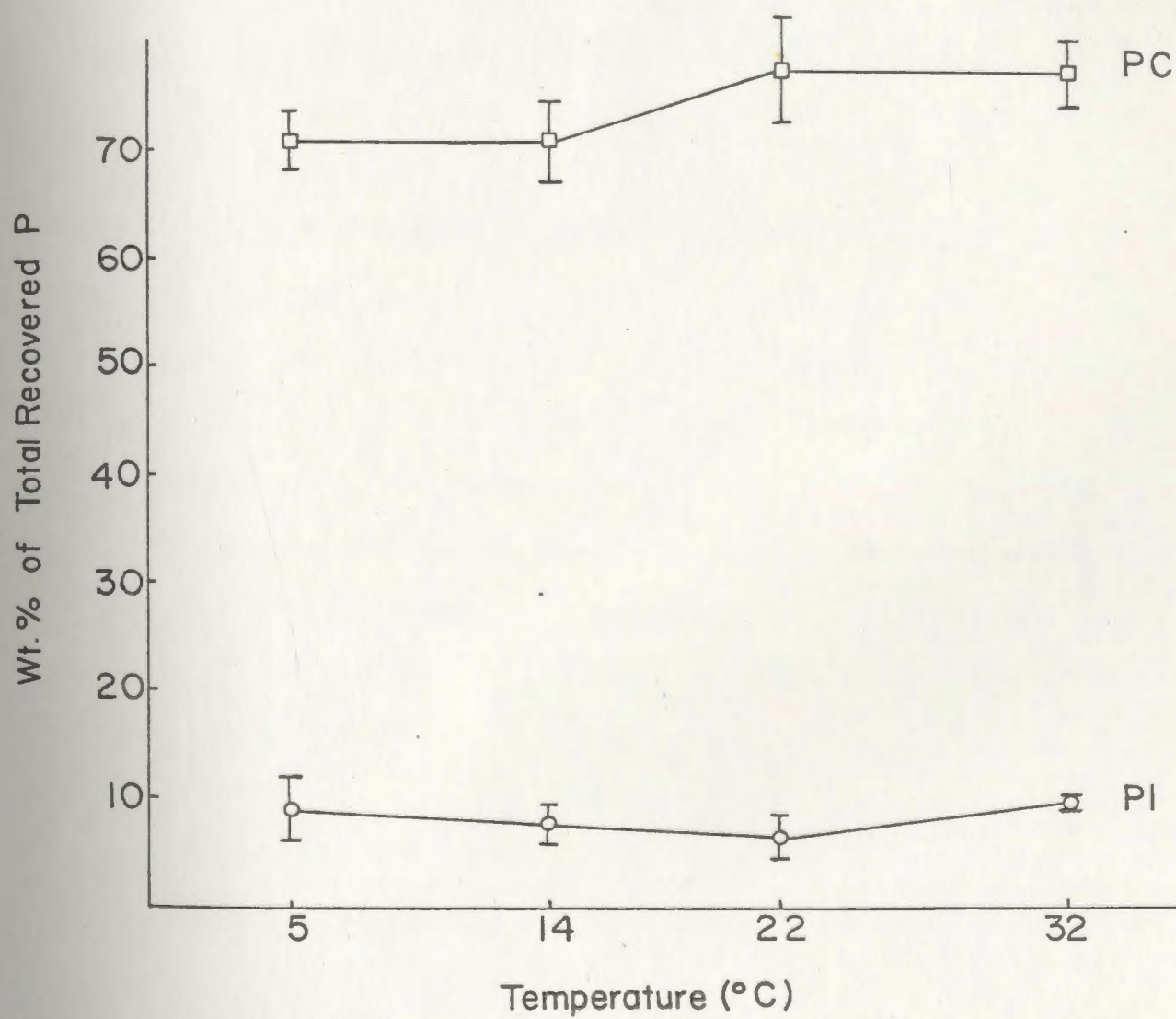


Figure 10: Phospholipids in surfactant that show significant changes with temperature. Error bars represent S.D.

of recovery was obtained by dividing the sum of the individual phospholipids by the total phospholipid applied. In the phospholipid analysis, the absolute recovery of applied P was $85.9 \pm 10.5\%$ for lung and $78.7 \pm 10.9\%$ for surfactant. As the results in Table 2 indicate, there were three major phospholipids in the lung tissue; namely, PC, PE and SM with relative weights about 40, 27 and 16% respectively. In surfactant, PC was the major phospholipid with relative weight of 71-78% over the temperature range as shown in Table 3. The phosphorus measurements of phospholipids other than PC fell at the lower limits of Bartlett determination and this might contribute to the slightly poorer recovery of lipid P in these preparations.

The unidentified fractions in the phospholipid analysis in both lung and surfactant represented phosphorus from more than one spot on the chromatogram. The fraction that ran with the solvent front in either solvent system contributed most to the value, this fraction was found to be ninhydrin positive in surfactant. Some phosphorus was also recovered from the gels at the origin. These spots were haphazard in occurrence even for samples obtained from turtles kept at one temperature, and the reason for this was not known. In samples from lung the phosphorus values from these unidentified spots remained quite constant at different temperatures, but there was a remarkable decrease of the surfactant counterparts at 32°C .

The relative weight of the phospholipids exhibiting significant changes with temperature is shown in Figs. 9 and 10. Although there were significant differences in PC and PE of lung with temperature, neither exhibited systematic changes. It is interesting to note that

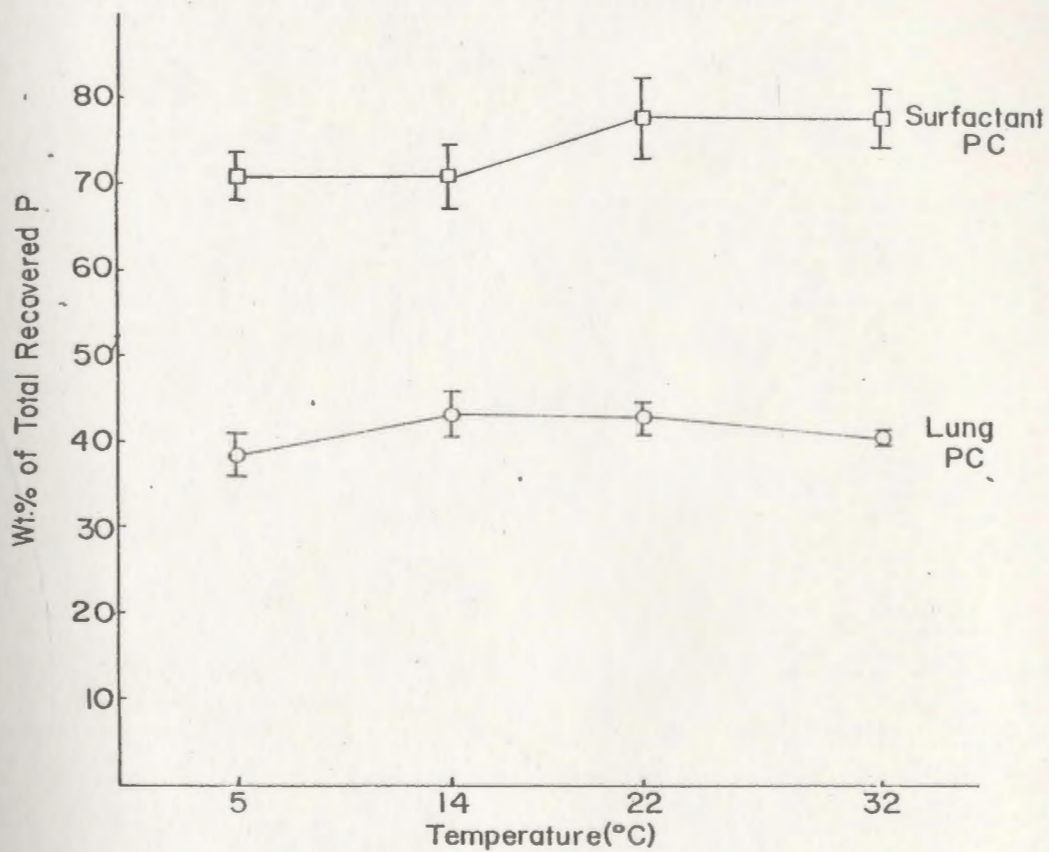


Figure 11: Content of PC in lung and in surfactant at different temperatures. Error bars represent S.D.

the increase of PC was accompanied by a concomitant decrease in PE to the same extent over the temperature range. On the other hand, there was a small but significant increase of PC and PI of surfactant at 32°C. The implication of these changes will be further discussed in a later Section. Fig. 11, compares changes of PC in lung and in surfactant from 5-32°C.

4. Fatty Acid Analysis of Total Lipids of Lung and Surfactant

In the following discussion, unless otherwise stated, the range of the mean values in lipids of either lung or surfactant from 5 to 32°C is denoted by two numbers connected by a dash '—'.

The monolayer phospholipid film studies have demonstrated that phases are dependent on both the phosphate head groups and the fatty acid chains (van Deenen, 1962; Phillips and Chapman, 1968; Träuble et al., 1974). Bialyers of phospholipid exhibit similar and related phase changes (see e.g. Lee, 1977a). It was therefore desirable to investigate the effect of temperature on the fatty acids of lipids of lung and surfactant.

Fatty acid analysis were usually carried out at 160°C for good separation of methyl ester of acids of chain length less than 20 carbon atoms, but preliminary analysis of lipid from animals at each temperature were carried out at 190°C to ensure that no significant quantities of long chain unsaturated acids were undetected. In these high temperature analyses a peak which had a retention time equal to that of cholesterol was obtained in total lipid of lung tissue but not in that of surfactant. This was in agreement with the report by Morgan et al. (1965) that homogenized dog lung had proportionately more cholesterol than washings from lung. In our study, cholesterol has not been quantified in either lung or surfactant.

The fatty acid profile of total lipid of lung as shown in Table 4a, has five major components: palmitate (19-24 mole%), palmitoleate (4-10 mole%), stearate (10-14 mole%), oleate (24-31 mole%) and arachidonate (14-20 mole%). In contrast, the major fatty acids in the total lipid of surfactant (Table 5a) were palmitate (35-44 mole%), palmitoleate (10-16 mole%), stearate (6-11 mole%) and oleate (19-23 mole%). When compared with the total lipid of lung, there was observed an enrichment of palmitate and palmitoleate in the total lipid of surfactant and a decrease of others, especially arachidonate, which was only 4-9 mole% of the total fatty acids in lipids of surfactant.

With the changes in environmental temperature, hence the body temperature, significant changes in a number of fatty acids were observed in the total lipid of both lung and surfactant. These changes were shown in Tables 4b and 5b, together with the P values from the student 't' test. For ease in observing potential trends and relationships between acids, the mole% of the fatty acids at different temperatures were plotted in Fig. 12 and 13. In total lipid of lung, both the short-chained fatty acids and the unsaturated ones decreased with increasing temperature and palmitate and stearate increased at 22°C and 32°C respectively, as might be expected if the physical state of cell membranes was important for permeability and other vital functions. The increase in oleate with increasing temperature was unexpected on the basis of physical properties alone. In contrast, no unusual changes were observed in total lipids of surfactant. Both stearate and palmitate increased and myristate and palmitoleate decreased with increasing temperature. The results confirmed the production of lipids with higher transition temperatures in response to increasing

Table 4a - Fatty Acid Composition of Total Lipid in Turtle Lung at Different Temperatures

Environmental Temperature °C	Number of Samples	Fatty Acid (mole %)															
		14 : 0	14 : 2 [*]	15 : 0	16 : 0	16 : 1	16 : 2 [*]	17	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 4	22 : 1 [*]	22 : 3 [*]	22 : 4 [*]
5	4	4.3 ± 0.9	2.5 ± 0.6	—	19.2 ± 1.9	9.6 ± 2.0	0.3 ± 0.5	0.9 ± 0.7	9.8 ± 1.0	24.5 ± 2.7	6.4 ± 1.8	0.7 ± 0.8	0.4 ± 0.5	20.1 ± 3.6	—	0.2 ± 0.3	1.4 ± 2.7
14	4	5.8 ± 2.3	2.5 ± 0.9	—	22.8 ± 3.7	10.0 ± 3.4	0.3 ± 0.6	0.8 ± 1.0	11.0 ± 1.1	24.1 ± 4.8	5.1 ± 2.3	1.3 ± 0.9	0.5 ± 0.3	15.5 ± 3.8	0.6 ± 1.2	—	—
22	4	4.5 ± 1.6	0.6 ± 1.3	—	24.1 ± 7.3	7.9 ± 2.9	0.3 ± 0.6	0.4 ± 0.7	11.0 ± 1.3	30.2 ± 4.2	4.3 ± 1.5	0.2 ± 0.4	—	15.8 ± 2.9	—	—	0.9 ± 1.8
32	4	2.4 ± 1.1	—	0.2 ± 0.5	21.3 ± 1.8	4.0 ± 0.3	0.2 ± 0.4	—	14.5 ± 0.5	31.1 ± 3.5	4.2 ± 1.5	3.6 ± 3.9	0.4 ± 0.7	14.2 ± 5.4	0.9 ± 1.2	—	1.7 ± 1.7

Values are means ± S.D.

— not detected.

* tentative identification.

4b - Summary of Significant Changes with Temperature

Fatty Acid	Temperatures	P
14 : 0	5 : 32	0.034
	14 : 32	0.036
14 : 2	5 : 32	<0.001
	14 : 32	0.002
16 : 0	5 : 22	0.016
16 : 1	5 : 32	0.011
	14 : 32	0.013
	22 : 32	0.040
18 : 0	5 : 32	<0.001
	15 : 32	0.001
	22 : 32	0.002
18 : 1	5 : 32	0.027

Table 5a - Fatty Acid Composition of Total Lipid in Turtle Lung Surfactant at Different Temperature

Environmental Temperature °C	Number of Samples	Fatty Acid (mole %)														
		14 : 0	14 : 1 ^a	14 : 2 ^a	15 : 0	16 : 0	16 : 1	16 : 2 ^a	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 1 ^a	20 : 4
5	4	5.0 ± 0.9	0.4 ± 0.8	0.7 ± 0.8	2.4 ± 1.4	35.2 ± 1.4	15.8 ± 1.6	1.3 ± 0.6	—	7.5 ± 0.4	21.7 ± 4.4	4.1 ± 2.2	0.6 ± 1.2	0.8 ± 1.6	—	4.6 ± 2.1
14	4	5.4 ± 2.1	1.0 ± 2.0	0.8 ± 1.6	1.5 ± 0.3	36.7 ± 6.0	12.9 ± 2.4	0.8 ± 0.9	0.9 ± 1.1	7.5 ± 0.7	19.2 ± 2.7	2.6 ± 1.7	1.7 ± 1.2	0.6 ± 1.2	—	8.5 ± 4.8
22	4	3.5 ± 2.1	0.2 ± 0.4	—	1.3 ± 0.2	43.1 ± 2.7	13.3 ± 3.0	1.0 ± 0.4	1.4 ± 0.1	6.4 ± 0.6	22.2 ± 2.2	3.5 ± 0.7	0.4 ± 0.9	—	0.4 ± 0.8	1.5 ± 1.5
32	4	2.3 ± 1.4	—	—	0.8 ± 1.7	44.4 ± 2.6	9.6 ± 1.1	—	—	11.2 ± 4.5	22.9 ± 2.8	3.3 ± 1.4	0.8 ± 1.0	—	—	4.8 ± 2.3

Values are means ± S.D.

— not detected.

^a tentative identification.

5b - Summary of Significant Changes with Temperature

Fatty Acid	Temperatures	P
14 : 0	5 : 32	0.015
	14 : 32	0.049
16 : 0	5 : 32	0.005
	5 : 22	0.011
16 : 1	5 : 32	0.001
	14 : 32	0.047
18 : 0	5 : 22	0.027

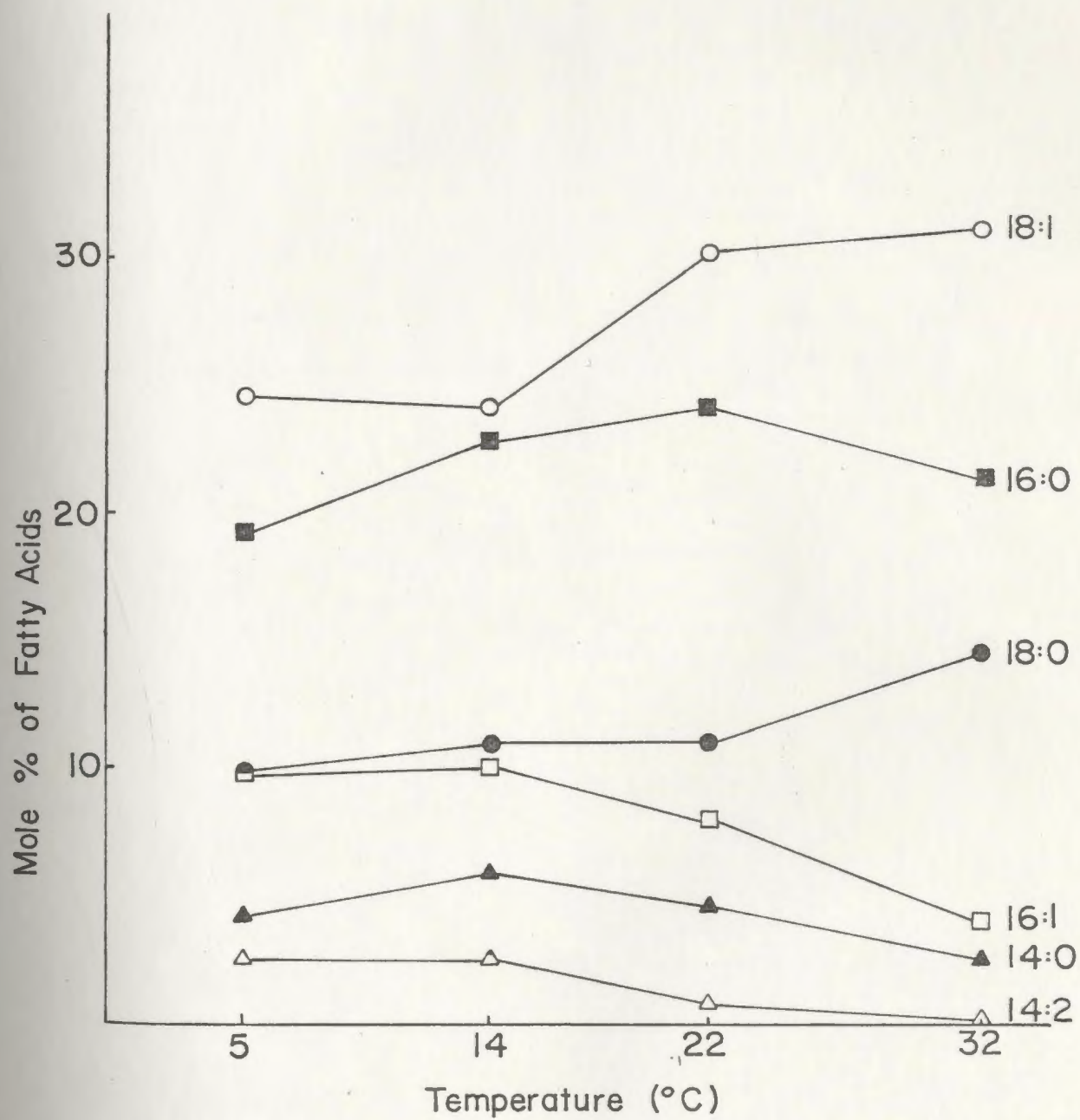


Figure 12: Fatty acids in total lipids of lung that show significant changes with temperature. S.D. omitted for clarity, see Table 4.

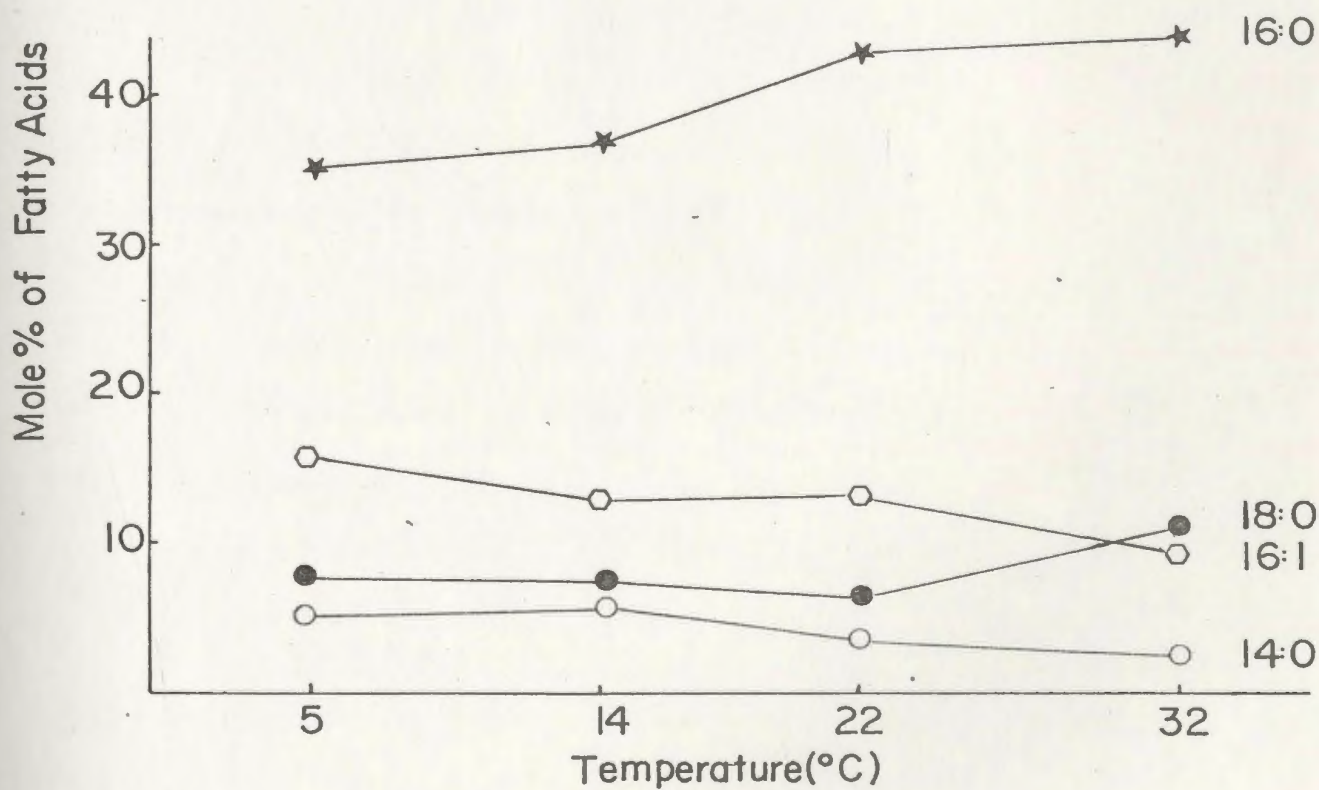


Figure 13: Fatty acids in total lipid of surfactant that show significant changes with temperature . S.D. omitted for clarity, see Table 5.

environmental temperature.

Since palmitate was quantitatively prominent in the total lipids of surfactant and DPPC in mammalian surfactant has been shown to be responsible for the lowering of surface tension (Clements, 1977), a more detailed treatment of palmitate data was warranted. Mole % palmitate in total lipid of lung and surfactant at different temperatures were plotted as in Fig. 14. As can be seen, although palmitate in total lipid of lung and of surfactant both showed significant changes with temperature, the changes in the latter were not reflective of general changes in the former, implying that there is a specific effect of environmental temperature on surfactant. Palmitoleate in total lipid of surfactant paralleled that of lung in that there was a concomitant decrease with increasing temperature as shown in the accompanying plot, (Fig. 14).

5. Fatty Acid Analyses of Individual Phospholipids in Lung and Surfactant

(a) PC in lung and PC in surfactant: The fatty acid profile of PC in lung as reported in Table 6a is, in general, similar to that of total lipid of lung, but significant increases in palmitate and palmitoleate and a reduction in arachidonate were evident in PC in lung compared to the total lipid of lung.

The fatty acid composition of PC in surfactant in Table 7a closely resembled that of total lipid of surfactant. The resemblance was not surprising as PC constituted more than 70% of the total phospholipid of surfactant. Palmitate was significantly enriched in PC in surfactant (49-57 mole%), compared to total lipid of surfactant (35-44 mole %) and there was less stearate in surfactant PC (3 mole %) than

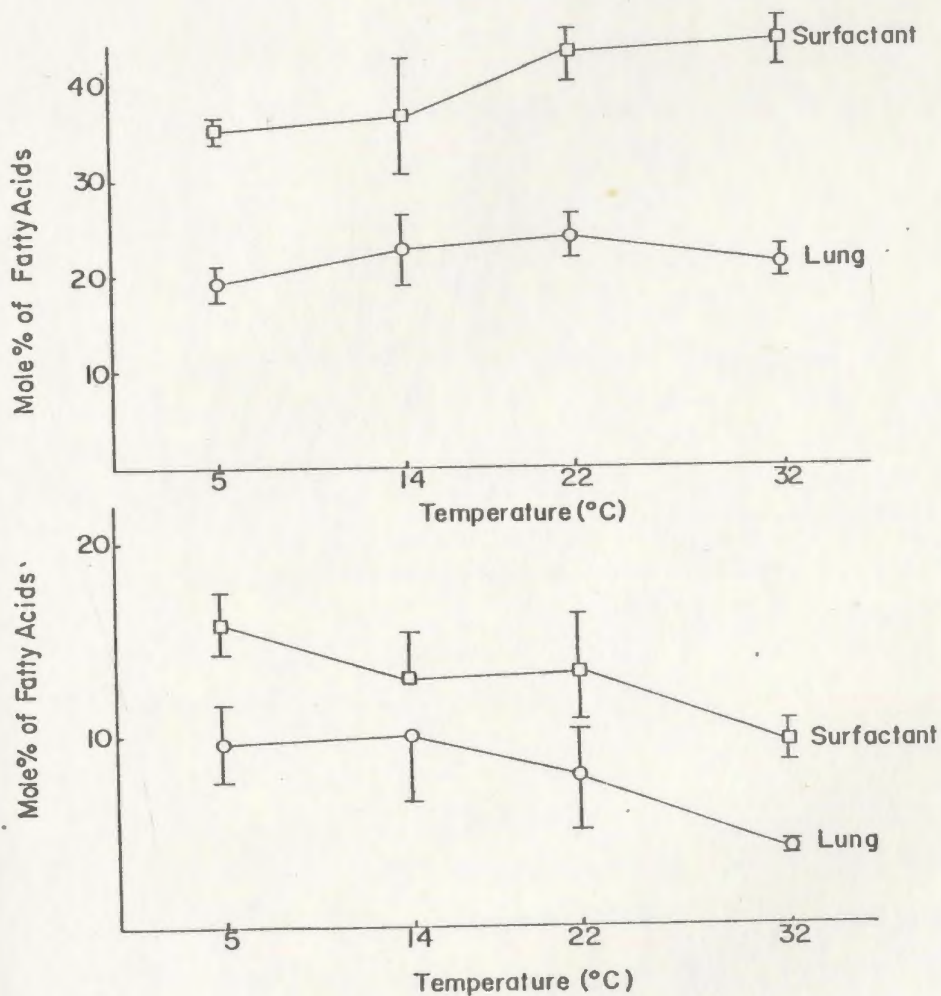


Figure 14: (Upper graph) Palmitate content in total lipid of lung and in total lipid of surfactant at different temperatures. (Lower graph) Palmitoleate content in total lipid of lung and in total lipid of surfactant at different temperatures. Error bars represent S.D.

Table 6a - Fatty Acid Composition of PC in turtle Lung at Different Temperatures

Environmental Temperature (°C)	Number of Samples	Fatty Acid (mole %)													
		14 : 0	14 : 2*	15 : 0	16 : 0	16 : 1	16 : 2*	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 4	22 : 1*
5	4	1.8 ± 0.3	0.6 ± 0.8	1.1 ± 0.3	28.4 ± 3.3	12.6 ± 1.6	1.1 ± 1.5	1.3 ± 1.6	6.3 ± 0.4	26.3 ± 1.8	6.9 ± 2.5	0.8 ± 1.0	1.0 ± 1.1	11.8 ± 1.6	0.2 ± 0.4
14	4	2.9 ± 1.0	—	1.4 ± 0.5	30.6 ± 2.6	12.3 ± 1.5	1.6 ± 1.2	0.7 ± 1.4	5.7 ± 2.1	25.6 ± 2.2	6.1 ± 2.0	1.5 ± 1.8	0.4 ± 0.9	11.4 ± 6.5	—
22	4	3.3 ± 1.9	0.2 ± 0.3	1.0 ± 0.4	35.7 ± 3.6	12.7 ± 4.3	0.6 ± 0.6	1.0 ± 1.0	4.6 ± 1.2	29.4 ± 2.5	3.9 ± 1.5	0.5 ± 0.5	—	7.2 ± 1.8	—
32	4	1.5 ± 0.3	—	0.9 ± 0.6	34.4 ± 2.6	6.2 ± 0.5	—	0.2 ± 0.5	7.5 ± 0.7	33.3 ± 2.9	3.8 ± 1.3	1.4 ± 1.2	—	10.8 ± 2.1	—

Values are means ± S.D.

— not detected.

* tentative identification.

6b - Summary of Significant Changes with Temperature

Fatty Acids	Temperatures	P
14 : 0	14 : 32	0.036
16 : 0	5 : 22	0.037
	5 : 32	0.028
16 : 1	5 : 32	< 0.001
	14 : 32	< 0.001
18 : 0	5 : 32	0.031
	5 : 22	0.033
	22 : 32	0.009
18 : 1	5 : 32	0.007
	14 : 32	0.006
20 : 4	5 : 22	0.015

Table 7a - Fatty Acid Composition of PC in Turtle Lung Surfactant at Different Temperatures

Environmental Temperature (°C)	Number of Samples	Fatty Acid (Mole %)											
		14 : 0	14 : 2 [*]	15 : 0	16 : 0	16 : 1	16 : 2 [*]	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 4
5	4	3.0 ± 0.5	0.6 ± 0.7	2.0 ± 1.2	49.0 ± 2.1	17.1 ± 2.1	1.1 ± 1.2	0.7 ± 1.4	2.9 ± 0.5	16.8 ± 3.4	2.5 ± 1.5	—	3.4 ± 1.2
14	4	4.3 ± 1.9	—	1.6 ± 0.5	46.5 ± 4.8	16.9 ± 2.7	1.0 ± 0.7	0.5 ± 1.1	2.8 ± 1.0	18.3 ± 1.8	2.9 ± 2.1	0.7 ± 1.5	4.5 ± 2.2
22	4	3.2 ± 1.8	—	1.5 ± 0.5	50.0 ± 4.6	15.8 ± 3.2	0.8 ± 1.0	1.4 ± 1.0	2.5 ± 0.8	19.9 ± 1.7	2.4 ± 0.9	—	2.7 ± 1.3
32	4	2.1 ± 0.1	—	1.1 ± 0.2	57.0 ± 1.0	12.0 ± 2.9	—	—	2.7 ± 0.6	19.2 ± 1.2	1.9 ± 1.3	2.2 ± 2.5	1.9 ± 0.4

Values are means ± S.D.

— not detected.

* tentative identification.

7b - Summary of Significant Changes with Temperature

Fatty Acid	Temperatures	P
14 : 0	5 : 32	0.012
	14 : 32	0.005
16 : 0	5 : 32	0.001
	14 : 32	0.005
16 : 1	5 : 32	0.030
	14 : 32	0.050
20 : 4	5 : 32	0.050

in total lipid of surfactant (6-11 mole %).

A comparison of the fatty acid compositions of PC in surfactant and that in lung revealed some similarity in the profiles. There was, however, a significant enrichment of palmitate in PC of surfactant which increased from 28-34 mole % in PC of lung to 49-57 mole % in PC of surfactant. Palmitoleate also increased from 6-13 mole % to 12-17 mole %. Significant decreases were found in stearate from 5-8 mole % in PC of lung to 3 mole % in PC of surfactant, in oleate from 26-33 mole % to 17-20 mole % and linoleate from 4-7 mole % to 2-3 mole %, and finally in arachidonate from 7-12 mole % to 2-5 mole %. The last observation was in harmony with the findings made by Morgan et al. (1965) who reported that arachidonate was present in whole lung lecithin in dog but only a trace was found in washings from lung. The enrichment in palmitate and palmitoleate in PC of surfactant relative to lung was suggestive of the importance of these fatty acid acyl chains in the functioning of the alveolar lining of the lung.

Both the palmitate and palmitoleate content of surfactant PC were found to change significantly with increasing temperature, the former increasing and the latter decreasing; the myristate and arachidonate content also decreased, as reported in Table 7b. The direction of these significant changes were all in keeping with the maintenance of the proper physical state at each temperature. The results of those acids showing significant changes were plotted in Fig. 16. The same statement can apply to the fatty acids of lung PC, with the exception of oleate which increased with increasing temperature. The significant differences were tabulated in Table 6b, and the mole % of fatty acids

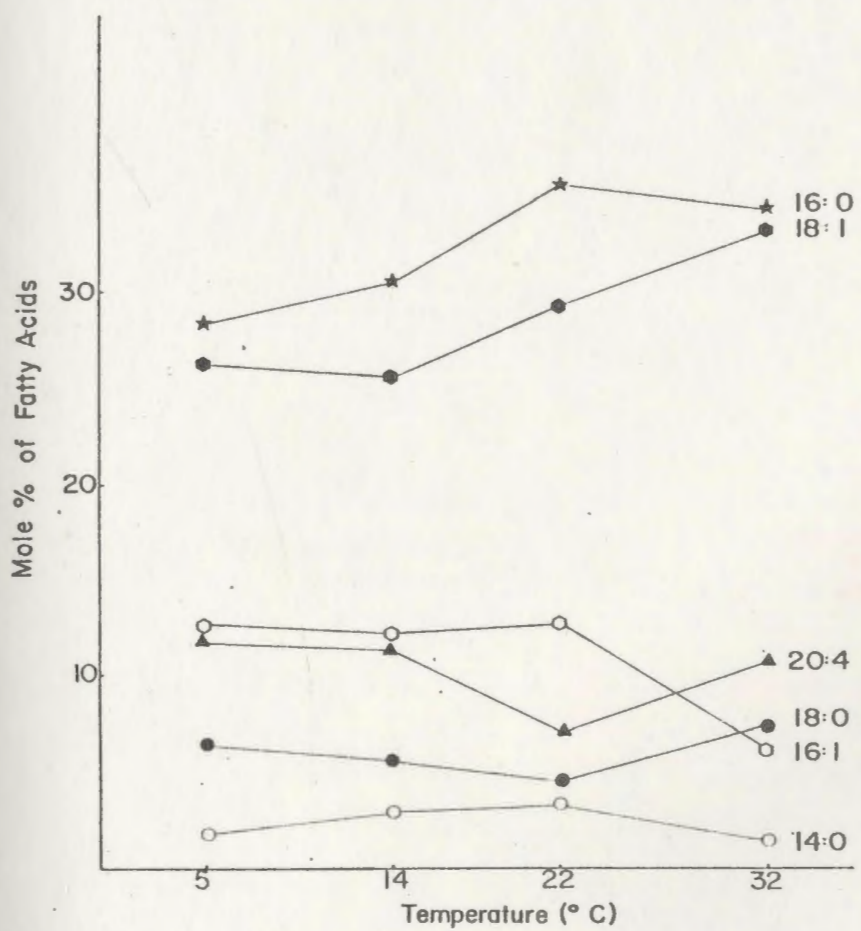


Figure 15: Fatty acids in PC of lung that show significant changes with temperature. S.D. omitted for clarity, see Table 6.

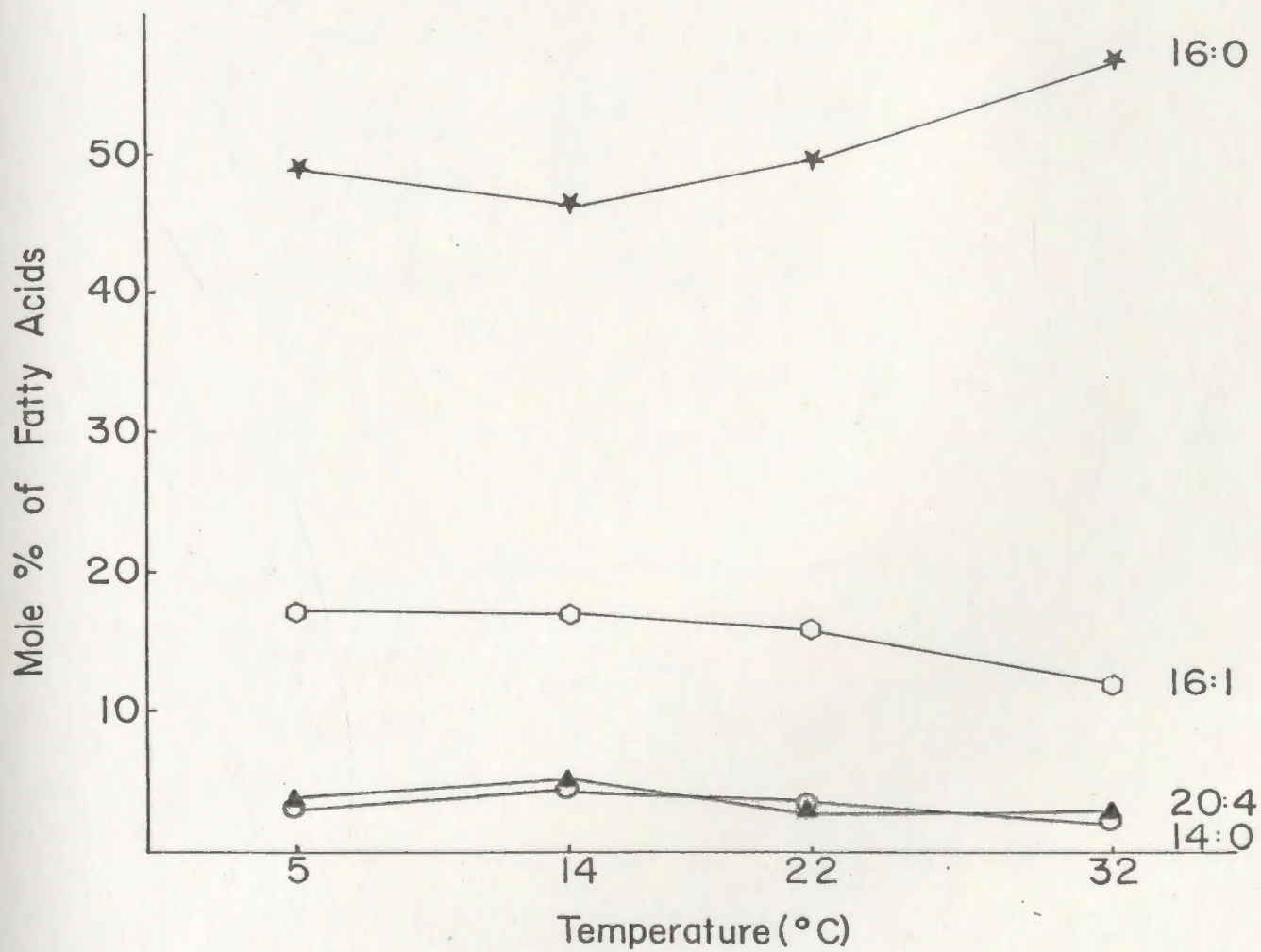


Figure 16: Fatty acids in PC of surfactant that show significant changes with temperature. S.D. omitted for clarity, see Table 7.

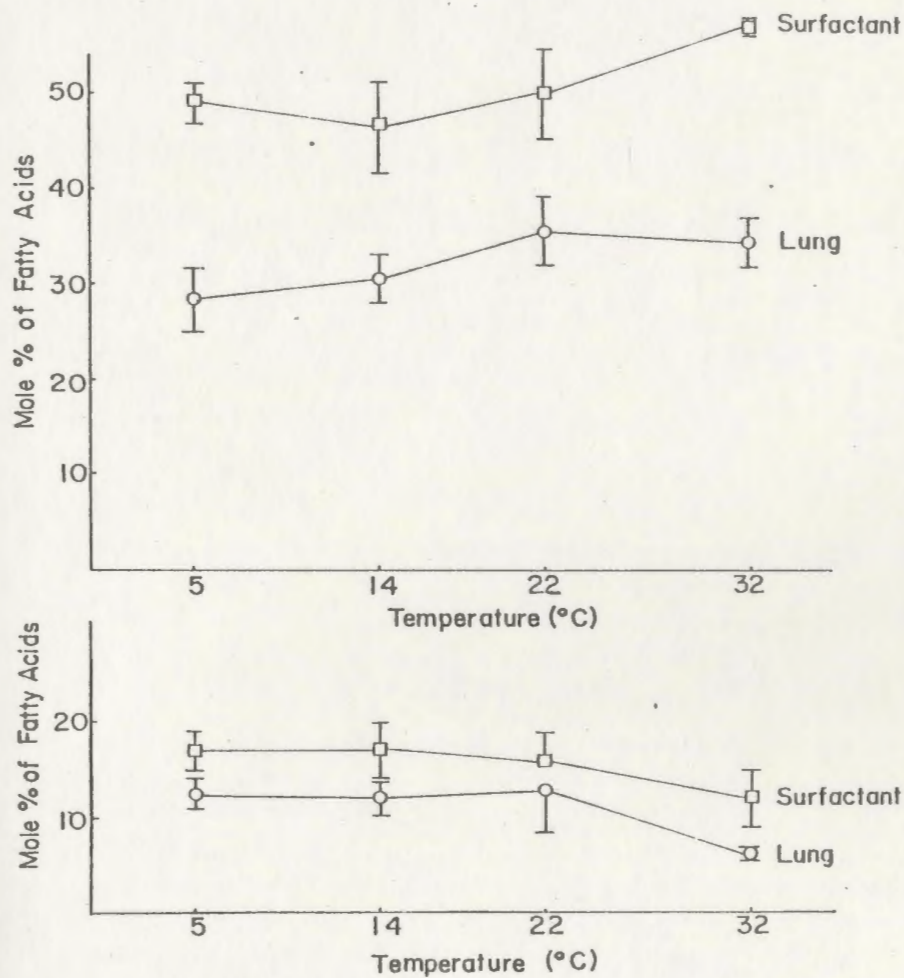


Figure 17: (Upper graph) Palmitate content in PC of lung and in PC of surfactant at different temperatures. (Lower graph) Palmitoleate content in PC of lung and in PC of surfactant at different temperatures. Error bars represent S.D.

in lung PC that showed significant changes at different temperatures were summarised in Fig. 15.

Fig.17 showed that the palmitate content of both lung and surfactant PC increased with increasing temperature. The patterns, however, were not parallel, again reflecting the differing effect of temperature on surfactant and lung. This result may be compared with the lower graph which showed a parallel decrease of palmitoleate in PC of both lung and surfactant.

(b) Lung PE and surfactant PE: PE was the second major phospholipid in the whole lung but comprised only a few percent by weight of phospholipid in surfactant. As mentioned in the previous section, the PE spot may represent PE and PG of lung, therefore, the reported numbers in Table 8a denoted mole % fatty acids from an unknown proportion of PE and PG. However, because of the location in the chromatogram corresponded more closely to PE standard than to PG, PE was expected to be the predominant lipid of the two. When chromatograms for the separation of phospholipids in surfactant were subjected to periodate-Shiff stain for glycolipids, PG was not detected.

The fatty acid profile of PE in lung was characterised by the preponderance of stearate 12-21 mole %, oleate 24-32 mole % and arachidonate 27-40 mole %. PE in surfactant had a fatty acid composition similar to that of PE in lung, but there was a relative enrichment in palmitate and palmitoleate and a relative reduction in stearate and arachidonate in PE of surfactant. Scatter in the results shown in Table 9 was considerable, and might be due to the small quantity present so that measurement at high sensitivity was subject to contamin-

Table 8a - Fatty Acid Composition of PE in Turtle Lung at Different Temperatures

Environmental Temperature °C	Number of Samples	Fatty acid (mole %)														
		12 : 0	14 : 0	14 : 2 ^a	15 : 0	16 : 1	16 : 2 ^a	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 4	22 : 1 ^a	22 : 2 ^a
5	4	0.2 ± 0.4	0.4 ± 0.5	2.2 ± 1.4	0.2 ± 0.3	7.8 ± 1.7	5.1 ± 0.8	0.9 ± 0.8	12.4 ± 0.6	24.5 ± 1.5	4.1 ± 0.9	1.0 ± 0.9	1.0 ± 1.2	39.6 ± 3.0	0.4 ± 0.8	0.3 ± 0.5
14	3	0.5 ± 0.9	0.7 ± 0.7	1.2 ± 1.6	0.3 ± 0.5	7.6 ± 0.6	8.4 ± 1.6	2.1 ± 1.2	16.5 ± 3.4	28.3 ± 3.2	4.4 ± 2.2	2.0 ± 1.9	0.4 ± 0.6	27.0 ± 11.2	—	—
22	4	—	2.1 ± 2.9	—	0.5 ± 0.9	8.1 ± 2.2	5.8 ± 2.6	0.9 ± 1.0	17.8 ± 4.0	32.4 ± 3.2	4.0 ± 2.4	0.3 ± 0.6	—	27.6 ± 11.0	—	—
32	4	0.5 ± 0.6	0.5 ± 0.6	—	3.8 ± 3.9	8.6 ± 2.6	1.3 ± 0.9	2.8 ± 3.1	20.7 ± 8.4	24.2 ± 4.4	1.4 ± 1.0	1.4 ± 1.9	1.8 ± 3.6	32.9 ± 3.7	0.2 ± 0.3	—

Values are mean ± S.D.

— not detected.

^a Tentative identification.

8b - Summary of Significant Changes with Temperature

Fatty Acid	Temperature	P
16 : 1	5 : 32	0.001
	14 : 32	0.001
	22 : 32	0.016
	5 : 14	0.013
18 : 1	5 : 22	0.004
	22 : 32	0.024
18 : 2	5 : 32	0.007
20 : 4	5 : 32	0.030

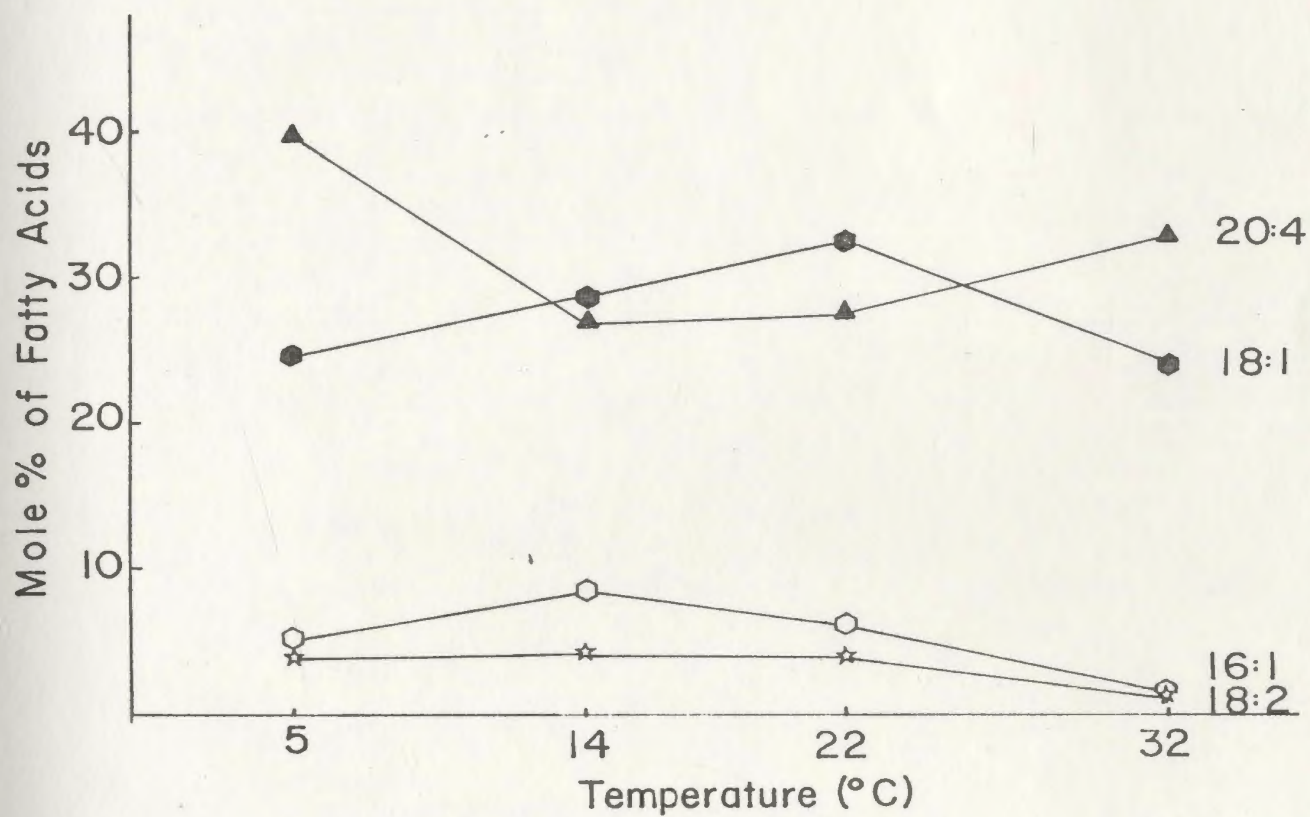


Figure 18: Fatty acids in PE of lung that show significant changes with temperature. S.D. omitted for clarity, see Table 8.

Table 9a - Fatty Acid Composition of PE in Turtle Lung Surfactant at Different Temperatures

Environmental Temperature °C	Number of Samples	Fatty Acid (mole %)																
		10 : 0	12 : 0	14 : 0	14 : 2 ^a	15 : 0	16 : 0	16 : 1	16 : 2 ^a	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 1 ^a	20 : 4	22 : 2 ^a
5	4	—	0.4 ± 0.5	4.0 ± 3.0	—	4.5 ± 3.6	23.0 ± 14.1	7.6 ± 4.5	3.7 ± 4.2	1.4 ± 2.8	9.9 ± 0.9	24.3 ± 6.6	3.5 ± 3.3	0.4 ± 0.7	0.3 ± 0.6	—	17.2 ± 12.9	—
14	4	—	0.5 ± 1.0	1.5 ± 1.0	2.7 ± 3.1	1.0 ± 1.1	14.3 ± 5.8	9.7 ± 9.7	—	3.6 ± 0.4	12.4 ± 3.6	31.7 ± 1.8	3.2 ± 2.1	3.0 ± 2.5	—	4.3 ± 7.6	12.6 ± 7.8	—
22	3	—	0.9 ± 0.8	3.9 ± 1.4	—	3.1 ± 1.1	12.2 ± 2.4	5.3 ± 1.2	3.4 ± 5.9	0.7 ± 1.2	14.2 ± 3.2	36.5 ± 5.9	6.5 ± 5.4	0.8 ± 1.3	—	—	10.2 ± 10.4	2.2 ± 3.9
32	3	5.2 ± 4.9	4.3 ± 2.0	7.4 ± 1.2	—	4.7 ± 2.3	14.3 ± 3.7	6.8 ± 3.0	2.4 ± 1.1	—	10.0 ± 1.9	27.3 ± 10.7	0.7 ± 0.8	2.9 ± 5.0	—	—	11.9 ± 7.6	—

Values are means ± S.D.

— not detected.

^a tentative identification.

9b - Summary of Significant Changes with Temperature

Fatty Acid	Temperature	0
14 : 0	14 : 32	0.001
18 : 0	5 : 22	0.021

ation. The significant changes in the fatty acid content of lung PE were summarised in Table 8b and plotted in Fig. 18. The trend of changes conformed to an increase of saturation and decrease of unsaturation with increasing temperature.

The significant changes in the fatty acid content of PE in surfactant were summarised in Table 9b. Myristate increased from 14-32°C and stearate increased from 5-22°C.

(c) SM in lung and SM in surfactant: In Tables 10a and 11a are presented the results of fatty acids analyses of SM in lung and surfactant. The fatty acid profile of SM in lung was marked by the unusually high myristate content (18-29 mole %) and by the presence of fatty acids with long retention times. Palmitate was also a major fatty acid (23-38 mole %) and it showed a significant increase with increasing temperature. In addition, there was a decrease in the myristate content between 22 and 32°C (Table 10b).

SM in surfactant had nearly the same fatty acid profile as that in lung. As summarised in Table 11b, the myristate content was higher at 14 than at 22 and 32°C and the oleate content was higher at 22 than at 5 and 14°C.

(d) PS in lung and PS in surfactant

The prominent features of the fatty acid composition of PS in lung in Table 12a included the presence of stearate and oleate in appreciable quantities (42-46 mole % and 18-29 mole % respectively) and moderate amounts of palmitate and arachidonate. The significant changes over 5-32°C occurred in palmitoleate and oleate as summarised in Table 12b. While the former was lower at high temperatures, oleate actually was

Table 10a - Fatty Acid Composition of Sphingomyelin in Turtle Lung at Different Temperatures

Environmental Temperature °C	Number of Samples	Fatty Acids (mole %)																22 : 1 [*]	22 : 2 [*]	22 : 1 [*]	22 : 4 [*]
		10 : 0	12 : 0	12 : 1 [*]	14 : 0	14 : 1 [*]	15 : 0	16 : 0	16 : 1	16 : 2 [*]	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 4				
5	3	—	1.9 ± 1.2	—	24.6 ± 3.2	2.9 ± 0.4	0.3 ± 0.5	23.9 ± 8.2	0.9 ± 0.8	0.8 ± 1.4	1.2 ± 0.3	5.4 ± 1.3	3.2 ± 1.0	0.6 ± 1.0	5.9 ± 5.1	4.9 ± 1.6	4.1 ± 1.2	0.7 ± 1.2	5.0 ± 5.1	1.7 ± 2.9	12.1 ± 2.4
14	2	—	2.6 ± 1.1	1.1 ± 0.6	26.4 ± 1.4	1.7 ± 2.3	—	22.6 ± 5.1	1.9 ± 0.1	—	3.2 ± 1.3	6.8 ± 1.3	2.4 ± 0.8	1.2 ± 0.7	3.1 ± 4.4	2.1 ± 2.9	4.1 ± 1.8	2.1 ± 3.0	1.2 ± 1.6	3.1 ± 4.4	14.5 ± 1.0
22	4	0.2 ± 0.4	2.0 ± 0.5	—	28.6 ± 3.4	0.9 ± 1.8	2.3 ± 2.9	37.5 ± 13.5	0.8 ± 0.9	0.9 ± 1.9	1.6 ± 0.9	5.0 ± 1.2	2.8 ± 3.1	0.1 ± 0.3	5.0 ± 4.3	2.9 ± 5.8	2.8 ± 2.1	—	1.9 ± 1.8	—	4.8 ± 0.5
32	4	1.4 ± 2.1	1.3 ± 1.3	1.7 ± 1.9	17.6 ± 4.6	—	3.6 ± 0.7	38.2 ± 5.4	0.4 ± 0.7	0.3 ± 0.6	0.3 ± 0.6	6.3 ± 2.3	2.1 ± 2.0	1.0 ± 1.5	8.1 ± 8.0	4.1 ± 4.8	3.9 ± 2.8	1.4 ± 1.6	—	—	8.2 ± 10.0

Values are mean ± S.D.

— not detected.

^{*} tentative identification.

10b - Summary of Significant Changes with Temperature

Fatty Acids	Temperatures	P
14 : 0	22 : 32	0.008
	14 : 32	0.028
16 : 0	5 : 32	0.018

Table 11a - Fatty Acid Composition of Sphingomyelin in Turtle Lung Surfactant at Different Temperatures

Environmental Temperature (°C)	Number of Samples	Fatty Acid (mole %)																			
		10 : 0	12 : 0	12 : 1 ^a	14 : 0	14 : 1 ^a	15 : 0	16 : 0	16 : 1	16 : 2 ^b	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 4	22 : 1 ^a	22 : 2 ^a	22 : 3 ^a	22 : 4 ^a
5	4	2.2 ± 4.5	2.8 ± 3.2	1.8 ± 2.1	24.3 ± 11.3	2.4 ± 1.7	2.4 ± 1.6	28.7 ± 17.8	3.8 ± 1.5	—	0.4 ± 0.8	7.5 ± 3.1	5.6 ± 1.8	0.5 ± 0.9	2.7 ± 3.8	0.6 ± 1.1	9.1 ± 10.4	—	5.0 ± 11.1	—	—
14	3	1.2 ± 2.1	2.9 ± 1.1	0.3 ± 0.5	31.8 ± 3.3	2.2 ± 0.5	1.6 ± 1.5	17.9 ± 4.0	1.8 ± 0.6	—	1.6 ± 1.0	4.6 ± 0.9	2.7 ± 1.1	0.3 ± 0.5	1.1 ± 0.9	7.8 ± 10.0	4.4 ± 1.5	5.4 ± 4.7	1.3 ± 2.3	1.4 ± 2.5	4.8 ± 8.5
22	2	—	3.1 ± 4.4	2.5 ± 3.5	12.4 ± 7.0	—	4.7 ± 2.3	23.5 ± 9.5	7.5 ± 3.3	—	—	11.5 ± 6.3	11.1 ± 2.1	1.0 ± 1.3	2.2 ± 3.0	—	2.5 ± 3.5	—	4.9 ± 6.9	5.0 ± 7.8	8.5 ± 11.2
32	4	—	6.2 ± 3.4	7.4 ± 6.9	19.2 ± 5.1	2.7 ± 1.8	3.5 ± 2.8	26.6 ± 8.0	5.9 ± 2.4	5.2 ± 10.4	—	5.8 ± 1.6	5.2 ± 3.0	3.7 ± 2.9	3.0 ± 3.5	0.5 ± 1.1	3.6 ± 5.3	1.6 ± 2.4	—	—	—

Values are means ± S.D.

— not detected.

^a tentative identification.

11b - Summary of Significant Changes with Temperature

Fatty Acids	Temperatures	P
14 : 0	14 : 22	0.022
	14 : 32	0.014
18 : 1	5 : 22	0.027
	14 : 22	0.008

Table 12a - Fatty Acid Composition of PS in Turtle Lung at Different Temperatures

Environmental Temperature °C	Number of Samples	Fatty Acid (mole %)																		
		10 : 0	12 : 0	12 : 1 ^a	14 : 0	15 : 0	16 : 0	16 : 1	16 : 2 ^a	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 1 ^a	20 : 4	22 : 1 ^a	22 : 2 ^a	22 : 3 ^a
5	4	—	0.9 ± 1.3	—	1.7 ± 1.4	0.4 ± 0.5	12.9 ± 8.9	4.1 ± 1.6	0.9 ± 1.0	1.1 ± 1.2	42.1 ± 7.5	18.1 ± 3.4	2.8 ± 2.3	2.2 ± 1.6	2.3 ± 1.9	0.8 ± 1.5	6.1 ± 2.7	1.4 ± 1.7	2.4 ± 2.8	—
14	4	—	0.4 ± 0.7	—	2.5 ± 3.2	0.4 ± 0.5	7.8 ± 2.1	4.3 ± 0.4	0.6 ± 0.7	0.8 ± 1.0	45.6 ± 4.9	22.0 ± 2.7	2.5 ± 2.0	2.1 ± 1.4	0.8 ± 0.6	1.4 ± 1.8	4.9 ± 4.0	0.2 ± 0.5	1.5 ± 1.1	—
22	3	0.9 ± 1.6	0.5 ± 0.9	0.3 ± 0.6	2.0 ± 0.3	0.5 ± 0.5	8.1 ± 3.9	3.2 ± 0.5	0.3 ± 0.5	0.6 ± 0.5	41.4 ± 6.5	28.5 ± 5.6	1.0 ± 1.1	1.6 ± 1.4	0.9 ± 0.8	—	6.4 ± 1.3	—	1.8 ± 1.1	1.9 ± 3.1
32	3	—	1.5 ± 1.3	2.1 ± 2.3	1.8 ± 1.1	0.8 ± 0.8	4.5 ± 1.1	1.1 ± 1.0	0.7 ± 1.3	0.3 ± 0.5	42.4 ± 3.1	27.5 ± 2.1	1.5 ± 1.9	3.0 ± 3.1	0.6 ± 1.0	—	10.6 ± 6.8	1.7 ± 1.6	—	—

Values are means ± S.D.

— not detected.

^a tentative identification.

12b - Summary of Significant Changes with Temperature

Fatty Acid	Temperature	P
16 : 1	5 : 32	0.034
	14 : 32	0.001
	22 : 32	0.024
18 : 1	5 : 22	0.027
	5 : 32	0.009

Table 13 - Fatty Acid Composition of PS in Turtle Lung Surfactant at Different Temperatures

Environmental Temperature °C	Number of Samples	Fatty acid (mole %)																	
		12 : 0	14 : 0	14 : 1 ^a	15 : 0	16 : 0	16 : 1	16 : 2 ^a	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 1 ^a	20 : 4	22 : 1 ^a	22 : 2 ^a	22 : 1 ^a
5	2	—	2.5 ± 3.5	—	0.8 ± 1.1	38.1 ± 36.4	7.4 ± 0.4	2.6 ± 3.7	2.5 ± 3.5	24.0 ± 6.8	11.1 ± 1.8	1.6 ± 2.2	1.6 ± 2.3	4.9 ± 6.9	—	3.3 ± 4.6	—	—	—
16	3	1.1 ± 1.8	3.6 ± 1.6	0.6 ± 1.0	1.2 ± 1.2	14.5 ± 1.9	5.2 ± 0.9	0.2 ± 0.3	2.8 ± 2.0	31.4 ± 6.4	20.7 ± 4.6	4.2 ± 4.0	6.3 ± 6.4	2.0 ± 0.8	1.0 ± 0.9	4.6 ± 2.3	0.6 ± 1.0	—	—
22	1	2.5	3.0	—	1.5	13.7	4.8	—	0.9	25.9	16.5	2.4	1.9	2.7	—	3.3	0.9	9.8	10.1
32	2	9.7 ± 9.4	5.2 ± 2.1	—	0.4 ± 0.6	10.9 ± 2.5	3.5 ± 0.2	—	—	36.7 ± 1.3	24.3 ± 1.1	2.1 ± 1.4	2.2 ± 3.1	—	—	3.6 ± 5.0	1.8 ± 2.5	—	—

Values are means ± S.D.

— not detected.

^a tentative identification.

lower at 5°C. We noted that oleate increased with rising temperature in several lung lipids, this one included.

The meagre quantity of PS in surfactant on many occasions denied detection by dichlorofluorescein spray. Hence not enough samples were collected for statistical analysis. From the available data in Table 13, it is observed that there was an enrichment in palmitate and a decrease in both stearate and oleate in PS in surfactant relative to that in lung.

(e) PI in lung and PI in surfactant

The fatty acid profile of PI in lung as reported in Table 14 was similar to that of PS in lung in that it showed high contents of stearate and oleate (38-52 mole % and 14-18 mole % respectively). Palmitate and arachidonate were present in moderate quantities. No significant changes were found.

The fatty acid composition of PI in surfactant in Table 15 resembled that of the corresponding lipid in lung, though there was a significant increase in oleate from 14-18 mole % in lung to 28-37 mole % in surfactant. It was found that the decrease of palmitoleate at 32°C was significant.

6. Saturated Fatty Acids as Mole % of Total Fatty Acids in Various Lipids

In the data presented above we have seen that the principal fatty acids in both the total lipid and its constituent lipids in surfactant were palmitate, palmitoleate, stearate and oleate. The pattern of changes was consistent with an increase in the saturated fatty acid chains with increasing temperature. In order to get at least some idea of the overall effect the increase of temperature had on various lipids,

Table 14 - Fatty Acid Composition of PI in Turtle Lung at Different Temperatures

Environmental Temperature °C	Number of Samples	Fatty Acid (mole %)															
		12 : 0	12 : 1 ^a	14 : 0	15 : 0	16 : 0	16 : 1	16 : 2 ^a	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 4	22 : 1 ^a	22 : 2 ^a
5	4	0.8 ± 1.0	1.5 ± 1.7	2.8 ± 2.7	0.9 ± 0.6	16.3 ± 10.8	3.4 ± 0.5	—	1.6 ± 1.4	38.1 ± 4.5	14.8 ± 2.9	4.8 ± 5.6	1.4 ± 2.7	0.5 ± 1.0	13.5 ± 13.3	—	—
14	3	1.2 ± 2.0	—	2.1 ± 2.0	1.1 ± 1.1	10.0 ± 0.7	2.4 ± 2.1	—	1.8 ± 1.7	51.5 ± 10.9	14.0 ± 3.3	4.3 ± 2.8	3.0 ± 5.2	—	9.1 ± 8.3	—	—
22	3	—	—	2.6 ± 1.2	2.2 ± 2.9	9.6 ± 4.5	2.3 ± 1.6	0.6 ± 0.7	1.1 ± 1.0	43.3 ± 17.6	17.9 ± 1.5	2.0 ± 2.0	2.5 ± 4.3	—	9.5 ± 15.7	2.7 ± 4.7	3.8 ± 6.6
32	4	2.1 ± 3.6	4.5 ± 5.3	5.0 ± 2.4	2.0 ± 2.0	11.2 ± 3.0	2.0 ± 2.3	1.1 ± 2.1	0.3 ± 0.7	41.3 ± 6.6	14.7 ± 2.6	4.8 ± 9.2	2.9 ± 5.8	—	8.2 ± 9.6	—	—

Values are means ± S.D.

— not detected.

^a tentative identification.

Table 15a - Fatty Acid Composition of PI in Turtle Lung Surfactant at Different Temperatures

Environmental Temperature (°C)	Number of Samples	Fatty Acid (mole %)																
		10 : 0	12 : 0	12 : 1 ^a	14 : 0	15 : 0	16 : 0	16 : 1	16 : 2 ^a	17 : 0	18 : 0	18 : 1	18 : 2	18 : 3	20 : 0	20 : 1 ^a	20 : 4	22 : 1 ^a
5	4	—	0.5 ± 1.1	1.9 ± 2.2	2.0 ± 1.5	0.6 ± 0.7	9.6 ± 7.7	3.9 ± 0.7	—	1.8 ± 1.8	41.0 ± 5.4	27.8 ± 8.3	4.0 ± 3.1	—	0.4 ± 0.8	—	6.6 ± 6.1	—
14	3	—	0.4 ± 0.7	—	1.2 ± 1.1	0.1 ± 0.6	6.0 ± 0.6	3.2 ± 0.7	0.2 ± 0.4	1.1 ± 1.0	36.6 ± 1.0	32.1 ± 6.6	5.2 ± 3.9	0.4 ± 0.4	—	0.4 ± 0.6	12.9 ± 6.2	—
22	4	—	1.0 ± 2.0	—	3.0 ± 3.1	—	5.4 ± 1.9	2.0 ± 0.8	0.8 ± 1.6	1.0 ± 0.9	47.9 ± 8.5	34.0 ± 10.8	1.4 ± 1.6	0.1 ± 0.3	—	—	3.1 ± 4.1	0.2 ± 0.3
32	4	2.0 ± 2.3	1.5 ± 2.5	—	1.3 ± 1.1	0.2 ± 0.4	6.7 ± 2.2	1.1 ± 0.8	—	—	44.6 ± 5.3	36.8 ± 4.8	2.3 ± 2.6	1.1 ± 2.2	—	—	2.6 ± 1.8	—

Values are means ± S.D.

— not detectable.

^a tentative identification.

15b - Summary of Significant Changes with Temperature

Fatty Acid	Temperatures	P
16 : 1	5 : 32	0.002
	14 : 32	0.016

Table 16 - Mole % Saturated Fatty Acid in Total Lipid
of Lung and Surfactant at 5-32°C

Environmental Temperature (°C)	No. of Samples	Saturated Fatty Acid (mole %)	
		Lung	Surfactant
5	4	34.5 \pm 3.6	50.9 \pm 2.9
14	4	40.8 \pm 4.7	52.4 \pm 6.4
22	4	39.9 \pm 3.2	55.6 \pm 3.9
32	4	38.7 \pm 3.1	58.6 \pm 2.1

Values are means \pm S.D.

Significant Changes

Lipid	Temperatures	P
Total Surfactant Lipid	5 : 32	0.005

Table 17 - Mole % Saturated Fatty Acid in Lung PC
and Surfactant PC at 5-32°C

Environmental Temperature (°C)	No. of Samples	Saturated Fatty Acid (mole %)	
		Lung PC	Surfactant PC
5	4	39.8 \pm 1.7	57.5 \pm 2.4
14	4	41.6 \pm 4.5	55.7 \pm 4.3
22	4	45.6 \pm 2.4	58.6 \pm 3.0
32	4	44.5 \pm 2.5	62.8 \pm 0.8

Values are means \pm S.D.

Significant Changes

Lipid	Temperatures	P
Surfactant PC	5 : 32	0.005
	14 : 32	0.048
	22 : 32	0.035
Lung PC	5 : 22	0.013
	5 : 32	0.02

Table 18 - Mole % Saturated Fatty Acids, in Lung PE
and Surfactant PE at 5-32°C

Environmental Temperature (°C)	<u>Saturated Fatty Acids (mole %)</u>	
	Lung PE	Surfactant PE
5	22.1 \pm 2.5 n = 4	43.4 \pm 14.0 n = 4
14	26.4 \pm 7.3 n = 3	32.8 \pm 3.7 n = 4
22	29.1 \pm 9.3 n = 4	35.1 \pm 2.8 n = 3
32	35.9 \pm 5.0 n = 4	45.8 \pm 10.0 n = 3

Values are means \pm S.D. n = no. of samples.

Significant Changes

Lipid	Temperatures	P
Lung PE	5 : 32	0.003

Table 19 - Mole % Saturated Fatty Acid in SM in
Lung and in Surfactant at 5-32°C

Environmental Temperature (°C)	Saturated Fatty Acid (mole %)	
	Lung SM	Surfactant SM
5	62.1 \pm 5.9 n = 3	49.1 \pm 36.1 n = 4
14	63.7 \pm 5.6 n = 2	69.3 \pm 2.6 n = 3
22	80.1 \pm 12.8 n = 4	55.1 \pm 24.9 n = 2
32	72.8 \pm 14.1 n = 4	61.8 \pm 11.1 n = 4

n = no. of samples.

Values are means \pm S.D.

Table 20 - Mole % Saturated Fatty Acids in PS in
in Lung and in Surfactant at 5-32°C

Environmental Temperature (°C)	Saturated Fatty Acid (mole %)	
	Lung PS	Surfactant PS
5	61.3 \pm 9.5 n = 4	51.7 \pm 37.6 n = 3
14	58.2 \pm 6.3 n = 4	56.5 \pm 6.0 n = 3
22	55.0 \pm 5.4 n = 3	50.2 n = 1
32	51.8 \pm 6.5 n = 3	62.8 \pm 13.2 n = 2

n = no. of samples.

Values are means \pm S.D.

Table 21 - Mole % Saturated Fatty Acids in PI in
Lung and in Surfactant at 5-32°C

Environmental Temperature (°C)	Saturated Fatty Acid (mole %)	
	Lung PI	Surfactant PI
5	60.8 \pm 12.3 n = 4	41.7 \pm 31.2 n = 4
14	67.2 \pm 8.8 n = 3	45.7 \pm 3.8 n = 3
22	58.8 \pm 21.5 n = 3	58.3 \pm 14.0 n = 4
32	61.9 \pm 8.9 n = 4	56.2 \pm 7.8 n = 4

n = no. of samples

Values are means \pm S.D.

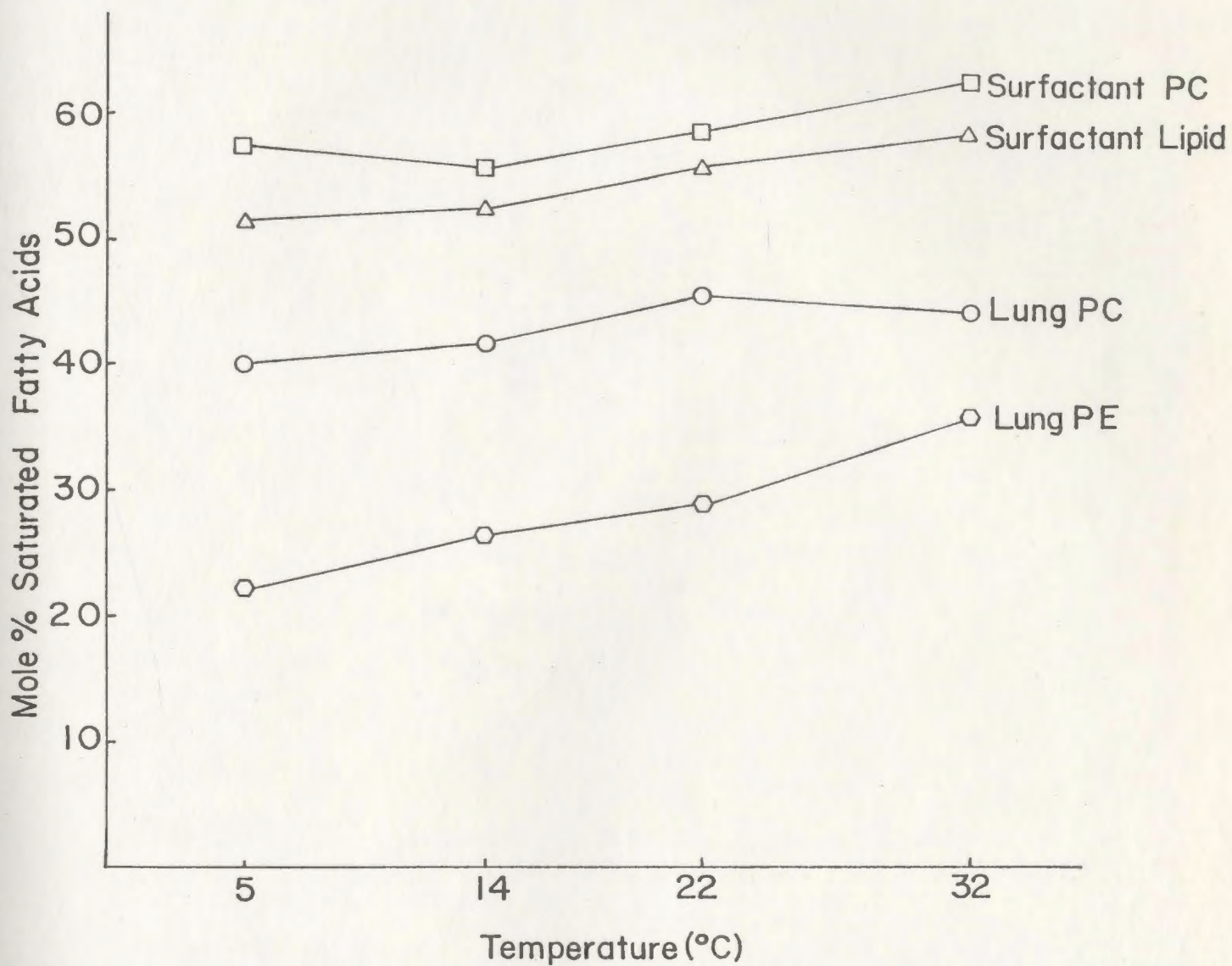


Figure 19: Lipids show significant changes in saturated fatty acid content with temperature. S.D. omitted for clarity, see Tables 16, 17 and 18.

the total saturated fatty acids were calculated for each lipid. The change in this number would give an indication of the general physical characteristics of that lipid at given temperatures. The results were shown in Tables 16 to 21. The lipids showing significant changes were summarised in Fig. 19. Total lipid of surfactant together with its major component PC showed significant increase from 5-32°C. Whereas, PC and PE in lung showed an increase in saturation with increasing temperature, the total lipid in lung did not.

It is interesting to note that saturated fatty acids made up less than 50 mole % of the total fatty acid contents in PC and PE in lung, PE and probably PI in surfactant. This may imply the occurrence of 1, 2-di-unsaturated phospholipids in turtle lung and perhaps surfactant. The significance of this will be further explored below.

7. The Estimates of Saturated PC as Mole Percent of Surfactant PC

The importance of DPPC in mammalian lung surfactant has been discussed previously, and the fatty acid composition of PC in turtle lung surfactant also suggested the presence of 1,2-disaturated PC. Therefore, an attempt was made to separate PC in surfactant into saturated and unsaturated fractions by cryochromatography. The fatty acid compositions of both fractions were determined.

The separations of a number of samples were not satisfactory. Some analyses were abandoned on the grounds of contamination when the unsaturated PC fraction showed enrichment of saturated fatty acids in comparison with the fatty acid composition of original PC in surfactant. Unfortunately, they could not be repeated because of a lack of sufficient lipid yield.

Nevertheless, some useful information has been gained from these

results. First, the saturated lecithin spot contained primarily palmitate (~85%) and this was independent of the environmental temperature of the animal. The other acids present were stearate 6%, myristate 5% and decapentanoate 4%. Thus the principal saturated lecithin was DPPC although other saturated lecithins with mixed acids also appeared to be present in small amounts.

Second, we have used the cryoplate results in three ways to determine if the saturated PC were changing with temperature.

(i) Based upon the amount of total lipid applied for the separation of PC on the original plate, the known PC content as a percentage of total lipid, and the fatty acid analyses of the saturated spot with internal standard, we obtained the minimum value for saturated PC content of lung lavage. This is shown in column A of Table 22.

(ii) For good separations, the unsaturated PC spot showed equimolar amounts of saturated and unsaturated acids. Therefore a second estimate was obtained by adding excess saturated acids from the unsaturated spot to the saturated PC value to give the corrected amount of disaturated PC. This was expressed as a percentage of the total load based upon phosphorus applied as in (i). The values were shown in column B of Table 22.

(iii) The third estimate was obtained by correcting the unsaturated PC, obtaining corrected values for saturated and unsaturated PC and calculating

$$\frac{\text{Corrected saturated PC}}{\text{Corrected saturated PC} + \text{corrected unsaturated PC}} \times 100$$

Values so derived are shown in column C of Table 22. These values provided an estimation of the minimum (A) and maximum (C) values for disaturated PC. For comparison the calculated maximum saturated PC,

Table 22a - Estimates of Mole % Saturated PC as % Of Total PC in Surfactant

Environmental Temperature ($^{\circ}\text{C}$)	<u>Cryochromatography Results</u>			
	Corrected Estimates A	Corrected Estimates B	Corrected Estimates C	Potential Estimated from Surfactant PC Profile D
5	2.6 ± 0.3 n = 2	3.5 ± 0.8 n = 2	17.4 ± 0.2 n = 2	15.0 ± 4.8 n = 4
14	2.1 ± 0.8 n = 2	3.2 ± 0.3 n = 2	12.9 ± 5.0 n = 2	11.4 ± 8.6 n = 4
22	6.8 ± 3.4 n = 2	8.4 ± 3.4 n = 2	26.6 ± 10.4 n = 2	17.1 ± 6.1 n = 4
32	4.2 ± 2.8 n = 3	9.0 ± 0.5 n = 3	30.9 ± 4.8 n = 3	25.6 ± 1.6 n = 4

Values are means \pm S.D. mole % of surfactant PC.

n = Number of samples.

22b - Significant Changes

Saturated PC	Temperature	P
Potential	5 : 22	0.013
	5 : 32	0.006
	14 : 32	0.048
	22 : 32	0.036

based on the fatty acid profile of the original PC is shown in column (D). All showed a trend of increasing disaturated lecithin with increasing temperature. Since the fatty acid profile showed a significant increase in saturation (Column D) and the saturated lecithin spot of the cryochromatogram exhibited a fatty acid composition which was consistent with DPPC being the major disaturated lecithins at all temperatures, we concluded that DPPC was increasing with increasing temperature.

8. Phase transitions of Phosphatidylcholine Mixtures Which Simulated PC in Surfactant

We have reported a number of significant changes in lipids of lung surfactant with temperature, which would be consistent with changes in the physical properties of the lipids. With such analytical data in hand it is desirable to carry out a detailed physical chemical investigation of lung surfactant in turtles and its constituent lipids. The very small yields of surfactant from turtles would necessitate a large outlay of time, effort and animals to obtain sufficient materials for detailed physical studies on turtles kept at all the four temperatures. As a preliminary step, we undertook a simple differential calorimetric study of some mixed lecithin systems designed to be approximately like PC in surfactant of turtles at different temperatures.

In this set of mixes, lecithin of turtle lung was purified by column chromatography and DPPC was added to it in known amounts. The thermograms of these mixes dispersed in water can be seen in Fig. 20. Also included for comparison is the thermogram of an aqueous dispersion of whole lung surfactant of rat. PC of turtle lung alone showed a very uncooperative transition below 0°C. The addition of 15 mole % DPPC (on the basis of phosphorus) yielded a more cooperative but broad

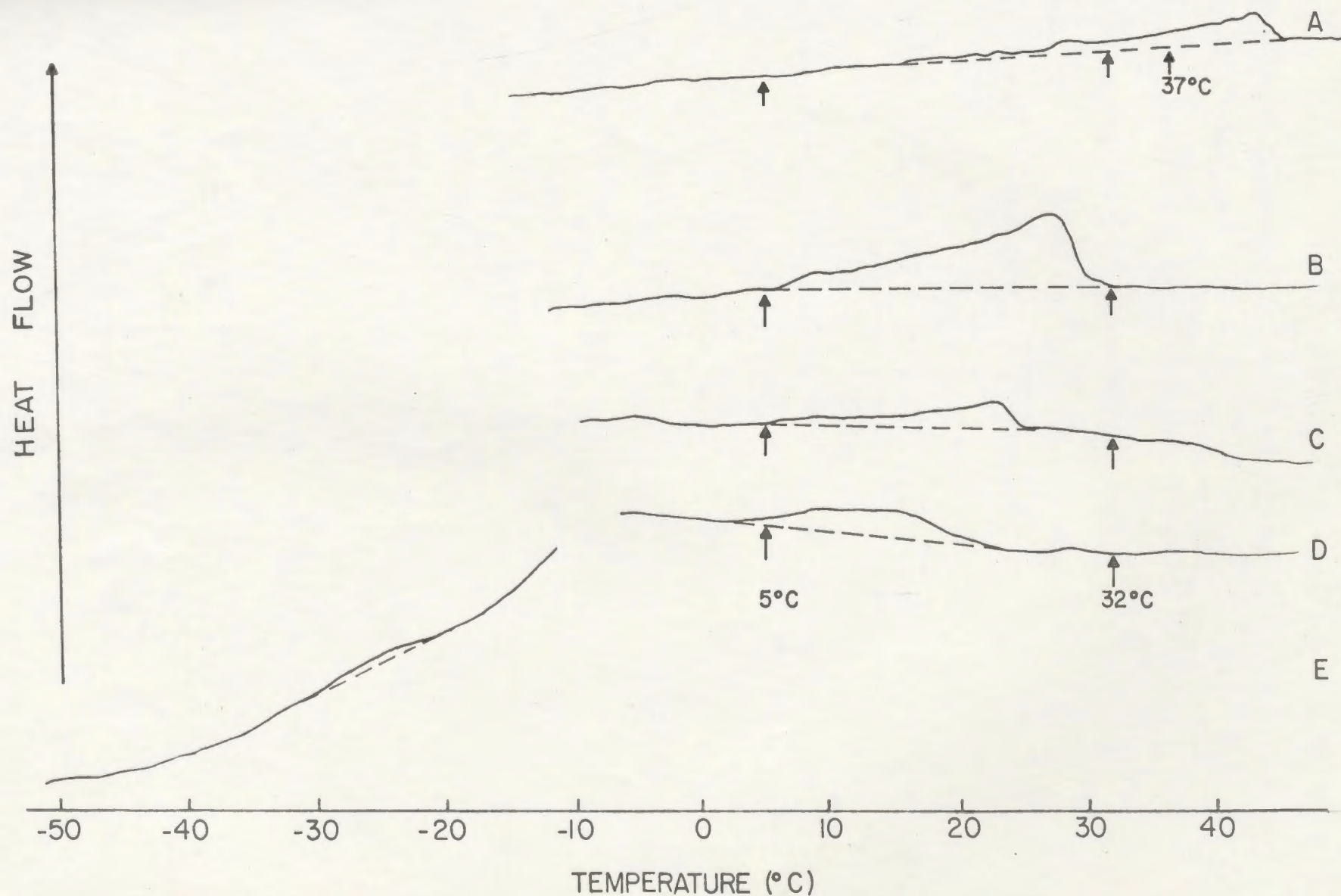


Figure 20: Effect of DPPC content on lipid phase transitions.

A: lavage fluid from rat lung; B: 40 mole % DPPC in PC of turtle lung; C: 29 mole % DPPC in PC of turtle lung; D: 15 mole % DPPC in PC of turtle lung; E: PC of turtle lung.

transition extending from 1 to 23°C. This was chosen as an approximation of the PC in surfactant from turtles acclimated to 5°C, based upon the data in Table 22 (Column D). Similarly, curve C would be approximately like PC in surfactant from turtles kept at 32°C based upon these data. If these were reasonable approximations of the PC in surfactant they confirmed the prediction of the analytical data that the position of the gel to liquid crystalline transition shifted upward with increasing DPPC. As is evidenced by the arrows indicating 5 and 32°C, the surfactant would be expected to exist in a mixed phase or be very close to the completion of the phase transition at the animal's body temperature. We note in this respect, that the lung surfactant of rat was also in a mixed gel-liquid crystalline at 37°C.

If one based the approximation of PC in turtle surfactant on the fatty acid composition, that is, enough DPPC was added to get the proper amount of palmitate as a percentage of the total, one obtained samples which exhibited thermograms C and B for turtles kept at 5 and 32°C respectively. Because of the excess unsaturation in PC of lung (see Table 17) we felt that the amounts of DPPC in these fractions were likely too high, but they nevertheless exhibited the same trend described above.

Discussion

1. Acclimation

In the study of metabolic responses of poikilotherms to environmental changes such as alteration of temperature, the times allowed before testing have varied a great deal among different researchers. Whether true acclimation has occurred in some of these studies undertaken over shorter periods is a moot point.

Smith (1967, 1976) has studied metabolic responses to temperature in the goldfish mucosa and found that many changes were relatively rapid, but in some cases while the major portion of the change occurred quickly, full acclimation might take some two to three weeks. With this in mind, a minimum acclimation time of four weeks was used in these studies. Three days was the acclimation period in the study of temperature effect on turtles by Jackson (1971). At least 2 weeks of acclimation prior to utilization was chosen in studying the effects of temperature on respiration of the red-spotted newts (Pitkin, 1977).

After injection of labelled palmitate, glucose or glycerol, Tierney et al. (1967) found that the half-life of PC in rat lung and DPPC, in particular, was 14 hr, while Mason (1976) using labelled choline found a half life of 45-46 hr. The biological half life value of palmitic acid-labelled and choline-labelled PC in alveolar wash of rabbit lung was found to be 16 and 30 hr respectively by Jobe (1977). Generally for a change of 10°C , the metabolic rate of unacclimated animals is altered by a factor of 2-3 (see e.g. Swan, 1974) and if there were no metabolic alteration to accommodate to temperature in our animals, half lives of 6-20 days may be expected for surfactant lipid in turtles at 5°C

(assuming a Q_{10} of 2 and the minimum and maximum estimates of half-life of lecithin at 37°C). If it were 20 days only about 60-70% of the lipid may have been replaced at 5°C at the end of one month. Nevertheless, ectotherms appear to have the ability to acclimate to temperature in such a way as to maintain the metabolic rate close to that obtaining at the preferred temperature of the animal (see e.g. Swan, 1974; Smith, 1976; Hochachka and Somero, 1973). Assuming such compensation to occur in turtles then adequate time for sufficient replacement should have been allowed at each temperature. In any case the turtles at 5°C were usually held for approximately two months to ensure complete adaptation.

Since the analytical data mentioned here indicated that changes did take place in surfactant lipids, it will now be useful to pursue this with detailed study of the time course of acclimation.

2. The Use of Lung Lavage Fluid to Study Changes Expected in Surfactant

In mammalian surfactant studies, various methods to purify lung surfactant have been developed (Frosolono et al., 1970; King and Clements, 1972a; Shelley et al., 1977). Although the procedures of some are more elaborate than others, these methods share a common feature in the use of either differential centrifugation or density gradient centrifugation or both to obtain surface active materials from lavage fluid and/or homogenate. The separation and purification are based on density differences, and great emphasis is placed on isolating quantitatively homogeneous fractions. The validity of these methods lies in the ability of the purified product to duplicate most of the accepted physiological functions of mammalian lung surfactant at 37°C , e.g. the lowering of surface tension to less than 15 mN/m and the rapid adsorption to the

surface of the subphase (King and Clements, 1972b). It is beyond question that these purification procedures are very useful in studying mammalian lung surfactant, but the nature of our study was such that it would not benefit from adopting these established purification techniques at the present stage. There were two reasons for this: (1) So far, the physiological functions of lung surfactant have been studied mostly at 37°C, and there are not enough data to tell what kind of surfactant the lung would need if it were to function at temperatures other than 37°C. Therefore, DPPC which is responsible for the lowering of surface tension, and which satisfies other physical criteria for lung surfactant at 37°C might not be playing as important a role at some other temperatures. In fact, this was one of the questions which was addressed in this study. Since the criteria for purification might need modifications, the purification procedure would have to change also. The formulation of the proper criteria would have to await further investigation (2) Any purification step would necessarily mean the elimination of some components in the lung lavage. As we did not have enough information about the functioning of lung surfactant in turtles we were reluctant to carry out purification by making a prior assumption about the chemical nature of the surfactant in map turtles.

It has been shown that lavage fluid contains less contaminating materials than does lung homogenate (Scarpelli and Colaccico, 1970; King and Clements, 1972b). It has also been shown that disaturated PC accounted for 55.5% of total phospholipid in lavage compared to 57% in highly purified surfactant (Oyarzün and Clements, 1976). Also the presence of unique protein components in purified mammalian lung surfactant has again been questioned (Maguire et al., 1977). It was suggested that

sedimentation as the purification procedure could lead to an artifactual enrichment of proteins (Shelley et al., 1975). The lavage certainly includes the totality of the surfactant and no components are reduced or enriched artificially. The estimation of lipid phosphorus in successive washings of turtle lung (Fig. 8) led to the conclusion that there was little contamination due to disruption of epithelial cells.

However, this is not to say that purification of lung surfactant of turtle is unnecessary in future studies. Perhaps the results of our present study might help to establish some guidelines for observing a purification procedure so that in addition to extracellular components, intracellular surfactant from turtle homogenate can be examined in later studies.

3. Lipid Phosphorus in Lung and Surfactant

It was found that lipid phosphorus in surfactant was 11-19 μ g of P/g of wet tissue over the entire temperature range. This was equivalent to approximately 0.3-0.5 mg ^{phospholipid} per g of wet tissue which was considerably lower than what has been found in rat 1.15 ± 0.1 mg per g of wet tissue (Toshima and Akino, 1972). However, the lung tissue, from rat had a higher phospholipid content as well, 20 mg per g of wet tissue (Toshima and Akino, 1972), when compared to 5 mg per g of wet tissue in turtle lung, so that the ratio of phospholipid from lung tissue to that from lavage in rat and in turtles were comparable. In rats, the ratio was 17, whereas in turtles it was 20-10, over the temperature range.

The increase in the phospholipid in surfactant with increased temperature could reflect a need for the production of extra surfactant because of increased work load in the lungs, or it might be concomitant with a change in lipid types. These points will be discussed more

extensively below.

4. Changes in Phospholipid Composition with Changes in Temperature

(a) Phospholipid composition in lung

(i) Comparison with other species: Table 23 compares the lung phospholipid of Malaclemys geographica in our study with that of Pseudemys elegans found by Harlan et al. (1966) and that of rat (Toshima and Akino, 1972).

In general, the values obtained by us and by Harlan et al. agreed well with the exception that a larger amount of sphingomyelin was found in our study. This might be due to some lyso PC which comigrated in our separation by thin layer chromatography. Dissimilarities in temperature and species may also explain the small differences. Samples from rat and turtle also showed a similar profile but the PC content was significantly higher and the PE content was lower in rat lung (body temperature 37°C) than in lungs of turtles.

(ii) Implication of changes: PC and PE were the major components of phospholipid in turtle lung and it is found that the trends of changes in PC and PE were complementary (Fig. 9). It has been shown from monolayer studies that the effect on the physical state of a particular PE monolayer by replacing the headgroup with choline is equivalent to raising the temperature by 20°C (Phillips and Chapman, 1968). Bilayer studies have also confirmed this by showing that aqueous dispersion of saturated PE undergoes gel to liquid crystalline phase transition at substantially higher temperature than PC (Chapman et al., 1974; Vaughan and Keough, 1974). Although the head group changes may be significant in terms of changes in membrane fluidity in the lung, they may more simply reflect a requirement for a maintenance of a constant amount of zwitterionic

Table 23 - Phospholipids of Lung from Various Species

Species	Body Temperature °C	No. of Samples	Phospholipid (wt% of total)						
			PC	PE	SM	Lyso PC	PS	PI	Unidentified
<u>Malaclemys</u> <u>geographica</u> (this study)	5	4	38.4 ± 2.4	29.7 ± 0.8	15.7 ± 3.2		9.5 ± 2.4	2.1 ± 2.4	4.9 ± 1.2
	32	4	40.1 ± 0.9	28.1 ± 1.8	17.4 ± 2.7		7.0 ± 1.9	2.8 ± 1.1	4.6 ± 3.1
<u>Pseudemys</u> <u>elegans</u> (Harlan <u>et al.</u> , 1966)	Unknown	8	47.5 ± 5.4	24.8 ± 7.7	8.7 ± 1.7	3.4 ± 3.7	13.2 ± 5.0		2.4 ± 2.3
Rat (Toshima and Akino, 1922)	37	5	52.3 ± 2.4	19.2 ± 2.6	7.6 ± 2.2	1.2 ± 0.1	5.4 ± 0.6	5.4 ± 0.5	8.5 ± 1.8

Values are means ± S.D.

head group in the lipids.

(b) Phospholipid composition of surfactant

Comparison with other species: Phospholipid composition of the lavage fluid in our study was compared with those of various species reported by other investigators (Table 24). The phospholipid profiles of surfactant in different species were surprisingly similar, which presumably reflects a need for material of a specific biochemical composition for proper surfactant function in all air-breathing vertebrates, and the preponderant PC content in all these species suggested the importance of lecithin in the structure and function of lung surfactant. In contrast to the finding that PG was a substantial component of mammalian surfactant and could be important in its function (Pfleger and Thomas, 1971; Godinez, 1975), PI was the second major component (6.5-10%) in lung surfactant of turtle. PG has been suggested to act to modulate the surface behaviour of mammalian surfactant (Hallman and Gluck, 1976). The same paper reported that PG was absent and PI was correspondingly prominent in surfactant from the preterm rabbit fetus. A comparison of the surface behaviour of surfactant from adult rabbit and preterm rabbit revealed that surfactant from both sources showed little difference in minimum surface tension, but whereas the compressibility of adult lung surfactant with PG peaked at surface tension 28-20 mN/m and fell at lower surface tensions, the fetal surfactant with PI had high surface compressibility whenever the surface tension was below 20 mN/m and continued close to the minimum surface tension (Hallman and Gluck, 1976). This difference was interpreted as indicating that PI was inferior to PG in stabilizing the film at 37°C since high compressibility is a measure of rate of

Table 24 - Phospholipids of Surfactant from Various Species

Species	Body Temperature °C	No. of Samples	Phospholipid (wt% of total)						
			PC	PE	SM	PG	PS	PI	Unidentified
Turtles	5	4	70.9 ± 2.7	5.4 ± 2.5	3.2 ± 0.5		2.1 ± 1.5	9.1 ± 2.9	9.4 ± 2.1
(this study)	32	4	77.1 ± 3.1	6.7 ± 1.9	3.5 ± 0.8		2.8 ± 0.2	10.0 ± 0.5	1.6 ± 1.9
Bullfrog (Hallman and Gluck, 1976)	—	—	76.3	6.4	0.8	9.1	1.5	4.5	1.0
Dog (Pfleger and Thomas, 1971)	37	—	74	4	5	10	2	3	1.3
Rat (Toshima and Akino, 1972)	37	5	68.4 ± 3.9	6.4 ± 1.8	3.8 ± 0.3	1.8 ± 0.4 (Lyso PC)	3.8	2.5	13.3

Values are means ± S.D.

loss of material from the surface film. Nonetheless the fact that chicken lung surfactant had no PG, and PI was the second major phospholipid, is in agreement with our results, and the evidence tends to imply that PI may replace PG in the functioning of surfactant. It is interesting to note that both PI and PG are acidic phospholipids with hydroxylated head groups and CDP-diglyceride serves as a common precursor for both. Further experiments on physical properties of PI at the appropriate lung temperature would be necessary to confirm its functional significance in lung surfactant. It is observed that both PC and PI increased significantly at 32°C. This is perhaps indicative of the importance of these components in meeting the specific physiological requirements at each temperature.

5. Fatty Acid Composition of Lung and Surfactant Lipids

(a) Total lipid in lung: While the fatty acids in total lipid of lung did show a number of significant changes over the temperature range, the pattern is not amenable to simple interpretation. On the basis of simple physical properties alone, the reduction in palmitate content and increase in oleate content with rising temperature (Table 4 and Fig. 12) were not expected. The values of oleate and palmitate could reflect some changes in neutral lipids with different effects on the transition temperature of the phospholipid bilayers (see e.g. Lee, 1977b), but we think this is unlikely as we also noted that similar changes occurred in these acids in PC of lung tissue. Perhaps the nonsystematic changes were due to the complexity of cellular functions involved in the lung as an organ. It is known that the mammalian lung has more than 40 different cell types (Sorokin, 1970), and if similar diversity in cell type were to be found in turtles, the great variety

of metabolic activities and functions of these cell types would necessarily require membranes of differing fluidity appropriate to their functions. Therefore a uniform effect of temperature on the lipids in lung would not be expected. Other acids which changed significantly (stearate, palmitoleate and myristate) showed changes which lead to more saturation at higher temperatures and thus are consistent with a change based upon a requirement for constant membrane fluidity.

(b) Total lipid in surfactant: In contrast, the total lipids exhibited changes in a pattern that is familiar to students in membrane research. Both major saturated fatty acids, palmitate and stearate increased with increasing temperature and unsaturated palmitoleate and short-chained myristate decreased as temperature was increased. At a low temperature, phospholipids with palmitate or stearate in the form of aqueous dispersion tend to form condensed, hence relatively rigid films, while phospholipids with short-chained or unsaturated fatty acids form expanded or relatively fluid membranes (see e.g. van Deenen, 1966; Chapman, 1973 and Papahadjopoulos, 1973). Monolayer studies have shown that shortening of chain length and unsaturation of the fatty acid constituents greatly increased the closest stable packing attainable (van Deenen, 1962) and the effect of temperature on the packing of the phospholipids was also demonstrated (Phillips and Chapman, 1968). It was found that the removal of 2 methylene groups from each fatty acid chain is equivalent to raising the temperature by 20°C. Also comparison of the data on the isotherms of different lecithin films obtained at room temperatures indicated that $C_{18:0}$, $C_{12:0}$ -PC gives an expanded film similar to that given by $C_{18:0}$, $C_{18:1}$ -PC (van Deenen et al., 1962). In bilayers, similar temperature and composition de-

pendent changes occur from gel to liquid crystalline states, (see e.g. Chapman, 1975; Lee, 1977a). The changes occurring to the total lipid of turtle surfactant can be interpreted as a response of the lipids to changing temperature to maintain the specific phases at each temperature.

The total lipid of lung and of surfactant might contain some neutral lipids and/or cholesterol, whose presence may help to modify the physical properties of the lipid layer(s). We noted in the results section that cholesterol was present in lung but seemed to be absent in surfactant. Determination of neutral lipid in surfactant was not carried out at this stage because of the lack of material. However we attempted reconstruction of fatty acid profiles of total phospholipid by appropriate summarization of the analytical data on the individual lipids. A paired t-test was performed to compare these calculated fatty acid composition with those of total lipid in lung and in surfactant. At 5°C the "reconstructed" fatty acid profile of phospholipid in surfactant was significantly different from total lipid of surfactant with respect to palmitate and palmitoleate. There was more palmitate in the "reconstructed" phospholipid (42.5% vs 35.2%) and less palmitoleate (14.5 vs 15.8%). At 32°C, linoleate content was lower in "reconstructed" phospholipid than in total lipid (1.9 vs 3.3%). Differences in total lipids and reconstructed phospholipids in lung were very small. It is true that this estimation did not provide a value for the absolute amount of neutral lipids present in surfactant, but it helped to indicate that neutral lipids are not present in very substantial amounts.

(c) PC in lung: The fatty acid profile of PC in lung had 2 major components, palmitate (28-36%) and oleate (26-33%). We found slightly

less palmitate and more oleate than did Harlan et al. (1966) who reported $39.7 \pm 14.1\%$ of palmitate and $21.0 \pm 6.2\%$ of oleate. The scatter in their values were considerable when compared with our results and it is tempting to speculate that the larger scatter in their results could be partly due to the varied environmental temperatures before the animals were used. As in total lipid of lung, the changes in general conformed to the increase of saturated and long chained fatty acids with increasing temperature. Oleate again stood out as an unsaturated fatty acid which increased with temperature. This anomalous behaviour of oleate has also been found in fungal membranes (Summer and Morgan, 1969). The reason behind it was not known, however, it could imply a need for a minimum number of double bonds in the lipid so that as linoleate and arachidonate were decreased, oleate increased in compensation. Such changes from polyenoic to monoenoic species with increasing temperature may also lead to slightly more rigid lipid.

(d) PC in surfactant: The fatty acids of PC in surfactant on the other hand, showed significant increase in palmitate. Palmitoleate, myristate and arachidonate all decreased with temperature, but only changes in palmitate and palmitoleate were of quantitative importance. This presents a clear picture of the phospholipid compensating for the effect of increasing temperature by increasing the relative proportion of saturated fatty acid on the one hand and decreasing the relative amount of unsaturated or short-chained fatty acids on the other so that the microviscosity in the phospholipid was maintained at a similar value. Such homeoviscous adaptation (Sinensky, 1974) has been shown in membranes of bacteria (McElhaney and Souza, 1976) and fish (Cossins and Prosser, 1978) but has not been obtained with lung surfactant

before.

(e) PE in lung and other lipids: The fatty acids associated with PE in lung were predominantly unsaturated. There were high contents of arachidonate, oleate, while palmitoleate and linoleate were found in smaller quantities (Table 8). The pattern of changes occurring to the fatty acids as a result of changing temperature was complicated (Fig. 18), and interestingly, these fatty acids were all unsaturated, probably reflecting that metabolic pathways of PE associated with unsaturated fatty acids were sensitive to temperature regulation.

Fatty acids of other phospholipids in lung were all found to be in good agreement with those reported by Harlan et al. (1966). There were no significant changes that occurred in fatty acids of PI in lung, the palmitoleate content of PI in surfactant decreased with temperature.

It is perhaps not too far-fetched to suppose that phospholipids which showed significant changes with temperature were involved in the phase-dependent functions of lung surfactant, more so than those that did not show changes. PC and PI were in this category and these phase-dependent processes will be discussed later.

6. Saturation of Lipids in Lung and in Surfactant with Temperature

This estimation was intended to show the overall physical state of each lipid at a particular temperature.

It is unfortunate that a quantitative analysis of the effect of say, myristate as opposed to oleate or linoleate on the overall fluidity of the membrane is not yet possible. Holub and Lands (1975) approached the problem in microbial mutant, Saccharomyces cerivisial KD46, by assigning a functionality factor to the unsaturated fatty acid of its membrane lipids. While this so called functionality factor is useful in

the context of their experiments and for the general understanding of membrane structure, it does not provide an absolute measure describing the effect of an individual fatty acid. Since the values which they pointed out to be helpful in assigning such a measure of fluidity in individual lipids such as compressibility and excess molal volume are not available for pure mixed acid lecithins, a measurement of the type used by Holub and Lands was not attempted.

It is possible, however, to get a quantitative estimate of the change in the saturated and unsaturated fatty acid content with temperature, which can be used as an index of changing fluidity in the surfactant and lung lipids.

For this estimation to be meaningful, one of the following conditions must be met by the lipids: (1) Only negligible amounts of short-chained saturated fatty acids should be present in that lipid. (2) These fatty acids with less than 14 carbons, if present should not change with temperature, or (3) if they did change, the change should be similar to that of unsaturated fatty acids, i.e. decreasing with increasing temperature. This is because short-chained fatty acids behaved physically like unsaturated fatty acids at a given temperature.

Except for sphingomyelin, most other lipids had very little fatty acids shorter than palmitate. In fact, since myristate decreased with temperature in total lipid in lung and in surfactant, PC in lung and in surfactant, the changes in physical state as denoted by the calculation of total saturation in these four lipids may have been underestimated.

Without exception, all the lipids that show significant changes with temperature exhibited an increase in saturation with increasing

temperature (Fig. 19). This could be taken to mean that phase was important to surfactant and also perhaps to lung cell membranes since surfactant total lipid and surfactant PC, lung PC and lung PE all tend to maintain the proper state of chain mobility by increasing the degree of saturation as temperature was increased.

Biological lipids usually have equimolar amounts of saturated to unsaturated fatty acids. Thus, excess of saturated fatty acids in surfactant PC indicated the presence of 1,2-disaturated PC which is also characteristic of PC in mammalian lung surfactant where DPPC constituted more than 50 mole % (see e.g. Träuble et al., 1974). The increase in the saturation reflected the increase in the disaturated PC species in surfactant as the temperature was elevated. This would be a mechanism adopted by the lung to maintain the physical state of surfactant when the environmental temperature was increased. Or perhaps one could say from an anthropocentric view, that the turtle lung surfactant functioned at low body temperature because of its ability to eliminate more rigid lipids.

Lipids that had less than 50 mole % saturated fatty acids necessarily consisted of diunsaturated fatty acids. Of these the major lung lipids PC and PE showed significant changes with temperature. PE in lung in particular had a phenomenal amount of diunsaturated fatty acids. The minimum estimation revealed that diunsaturated species might increase from 14 mole % to 22 mole % as temperature was decreased from 32 to 5°C. Turtle lung cells might adopt the regulation of the production of diunsaturated fatty acids a mechanism to preserve the microviscosity of the membrane when environmental temperature changes.

The two mechanisms, namely, regulation of production of disaturated

phospholipid and diunsaturated ones have been found to exist in E. coli. When the bacteria were incubated at 17 and 37°C for more than 10 minutes, they possessed very contrasting phospholipid molecular species profile (Kito, 1977):

Molecular	Temperature	
	17°C	37°C
Disaturated	2.3	11.3
-saturated- unsaturated	47.7	66.9
Diunsaturated	50.0	21.8

Also, the time course of incubation showed that when 37°C bacteria were incubated at 17°C, there was a gradual increase of diunsaturated fatty acid and a decrease of disaturated species (Kito, 1977). A more detailed analysis of the molecular species of lipids in turtle lung is needed to confirm the speculation.

It was also of interest to note that PE is the lipid in which very large amounts of unsaturated fatty acids were present. Since as noted above, PE shows equivalent physical behaviour at about 20°C higher than PC of the same chain length, it may be possible that the high unsaturation compensated for the head group. This might explain why PE was rich in polyenoic acids, especially arachidonate in comparison with the unsaturated acids in PC of lung (Tables 6 and 7). However, it must be conceded that the requirement for a PE head group may be dictated by other factors such as specific head group-protein interaction.

7. Direct Estimation of Saturated PC in Surfactant and Correlation with Physical Changes

The experimental determination of saturated PC in surfactant

(section III-7) showed that saturated PC in turtle surfactant consisted of predominantly DPPC, which increased with increasing temperature. The presence of large amounts of DPPC in mammalian surfactant is well documented and it would appear that the ability to make this unusual lipid is of unique evolutionary importance to lung (Clements et al., 1970). The fact that its content was temperature dependent, however, presumably underscored a requirement for specific physical state in surfactant at all temperatures and perhaps explained the high content of DPPC in mammalian pulmonary surfactant.

The data collected so far suggested that increased environmental temperatures induced increased production of saturated PC in turtle lung surfactant, primarily in the form of DPPC. This would have a profound effect on the physical state of surfactant lipids as the alveolar lining film becoming more liquid condensed to compensate for the increased molecular motion at high temperature.

The effect of DPPC on the physical state of PC in lung was demonstrated in the scanning calorimetric thermograms (Fig. 20). It is observed that by enriching PC in lung with DPPC, the transition from gel to liquid crystalline state of the lipid was caused to shift to higher temperatures. It is interesting to observe that lavage fluid from rat lung which presumably has a high DPPC content (Toshima and Akino, 1972) underwent a similarly broad transition, but peaked at 42°C. Träuble et al. (1974) reported broad transition also for purified surfactant from dog lung and PC in surfactant from 20 to 40°C. The physical meaning of this was that the lipid in rat surfactant at body temperature 37°C was in a mixed gel and liquid crystalline state. In other words, the surfactant lipid was possibly at a critical state at

the body temperature, so that a slight increase or decrease of the temperature could bring about a facilitative effect to change the lipid into liquid crystalline or gel phase respectively.

Our attempts at mimicking PC in surfactant of turtles kept at 5 and 32°C did not have such definitive results, but it is reasonable to conclude tentatively that the true surfactant may be at least close to, if not undergoing, a phase transition at the environmental temperature. Obviously, more experimental data on lung surfactant and PC itself in surfactant from turtles will be necessary before a definitive conclusion can be made.

8. Summary of Major Findings

With increasing environmental temperature, hence, body temperature, the following significant changes were observed in lipids of lung and surfactant of map turtles: (1) There was an increase of lipid phosphorus per g of fresh lung tissue in surfactant but not in lung. (2) In surfactant, PC increased as a proportion of the total lipid, PI showed anomalous behaviour, being higher at 5 and 32°C (~10%) and lower (~7%) at 14 and 22°C. The changes in PC and PE of lung tissue appeared to be complementary so that the total zwitterionic head groups content was always approximately the same. (3) Total lipid in surfactant showed a proportionate increase in its palmitate and stearate contents and decrease in palmitoleate, myristate and arachidonate contents. Total lipid in lung on the other hand exhibited non-systematic changes. (4) Palmitate content increased, and palmitoleate, arachidonate and myristate contents decreased proportionately in PC of surfactant. Saturated fatty acids of PC in lung increased and unsaturated and short-chained fatty acids

decreased with the exception of oleate which increased. (5) The percentage of saturated fatty acids increased in total lipid, in PC of surfactant and in PC and PE of lung. (6) There was an increase in saturated PC as a percentage of PC in surfactant. (7) Approximated surfactant PC exhibited broad gel to liquid crystalline transitions, which changed with DPPC content.

General Discussion

1. Temperature Induced Changes in Parameters Involved in Respiratory Physiology

It is recognized that variation of environmental temperature results in a number of changes in the parameters involved in the regulation of acid-base balance in ectotherms (see e.g. Howell and Rahn, 1976). Therefore an examination of these changes in relation to our findings and other available evidence is appropriate.

(a) Ventilation: Contrary to the general opinion that ventilation is a temperature dependent variable in turtles, it has been observed that pulmonary ventilation in terms of minute volume (\dot{V}_E) which is defined as expired volume of gas per minute, in fresh water turtles is independent of environmental temperature (Jackson, 1971; Jackson et al., 1974). In their reports the authors were able to show that the respiratory minute volume in Pseudemys scripta elegans kept at 10, 20 and 30°C for at least three days did not change significantly. Hence, although it has been shown that in mammals increased ventilation stimulates surfactant release (Wyszogroski et al., 1975, Oyarzün and Clements, 1977) and that elevated frequencies of ventilation stimulates glucose entry into the lung and palmitate utilization is accelerated to maintain essential lung lipids (Gassenheimer and Rhoades, 1974),

ventilation as a mechanism did not seem to be adequate in explaining changes in phospholipids observed in our experiment. There is a report by Lucey and House (1977) that temperature alters the type and number of ventilating sequences in turtle. They reported an increase in respiratory rate as temperature rose between 5 and 20°C but there was no consistent or significant change in respiratory rate between 20 and 38°C. Nevertheless, the experiment was carried out in anaesthetized animals which had been kept at the assigned temperature for 1 1/2 - 2 1/2 hr. Also since breathing became shallow as the rate increased, total \dot{V}_e would remain relatively unchanged. Furthermore, even if changed lung activity reduced the apneic periods (Lucey and House, 1977), which in turn caused increased surfactant secretion, there is no reason to expect that it would result in the change of the quality (i.e. saturation) of the lipids, especially in view of the results of Oyarzün and Clements (1977) who found the proportion of disaturated PC in lung lavage of rabbit lung to be constant even though the total phospholipid increased with ventilation.

(b) Increased carbon dioxide in the lung: Reeves, in his review article on the interaction of body temperature and acid base balance in ectothermic vertebrates (Reeves, 1977), summed up a huge body of information by stating that partial pressure of carbon dioxide (P_{CO_2}) in the arterial blood of ectodermic vertebrates increased with temperature in all species. The increase in the production of CO_2 (\dot{V}_{CO_2}) in the lung

of reptiles with increased temperature was also well documented (see e.g. Howell and Rahn, 1976). It is reasonable to ask if the increase in surfactant phospholipid was stimulated by an increase in P_{CO_2} and V_{CO_2} . Longmore and Mourning (1977) found that the increase of total CO_2 concentration from 7 to 43 mM at pH 7.35 in the medium perfusing isolated rat lungs resulted in an increase of $[U-^{14}C]$ -glucose incorporation into PC and PG in the intracellular and extracellular components of surfactant prepared from lung homogenate and in the remaining total lung lipids. $[U-^{14}C]$ -glucose incorporation into PC and PG of surfactant fraction increased by 43% and 22% respectively. However the increase in incorporation into the corresponding phospholipids of the lung fraction was greater; namely, PC by 50% and PG by 34%. Although they also reported an increase in the specific activity of palmitic acid in the surfactant PC of 56%, the increase in lung PC was again higher, at 77%. Our findings do not appear to be parallel to these results: The 10% increase in PC of turtle surfactant was gradual from 5-32°C. PC in lung did not show a consistent increase with temperature. Palmitate in PC in surfactant increased by 16% from 5 to 32°C but only 10% increase was observed for PC in lung. It was observed that the surfactant in our study represented only the extracellular fraction of total surfactant, but if we assume that the intracellular surfactant were affected by acclimation to the same degree as the extracellular counterparts, then the observed increase in lung lipids would have been overestimated. This consideration makes the results in the two studies look even more unrelated. Therefore, if the increased P_{CO_2} , resulting from increased environmental temperature, did stimulate the production of surfactant in turtles, the mode of action must have been quite

distinct from that in mammals. Since there is no reason to suppose such distinction should exist, it is perhaps proper to conclude that the effect of changing P_{CO_2} may have been responsible for some of the changes observed in our experiment, but the significance of this contribution is doubtful. It is noteworthy that in the P_{CO_2} determinations reported, the acclimation times were usually a matter of hours, it would be interesting to know if there had been any changes in P_{CO_2} for turtles acclimated for at least one month. Again we note that it is difficult to find any reason to assume that V_{CO_2} would cause a change in the saturation of surfactant lipid.

(c) Nutrition: It was noticed that the feeding habits of turtles were affected by environmental temperature. Turtles at $32^{\circ}C$ had by far the best appetite, $22^{\circ}C$ turtles ate a considerable amount but turtles at $14^{\circ}C$ and $5^{\circ}C$ failed to consume any detectable amounts of food. Thus, it could appear that we were comparing the lipid analyses in the lung and surfactant of the fed turtles with the starved ones by manipulating their body temperature. Before we so conclude, we must realise that the feeding habits of a healthy ectotherm is such that the food taken in is sufficient to meet its metabolic demand at that temperature. So long as the turtles at 5 and $14^{\circ}C$ looked as normal and healthy as those at 22 and $32^{\circ}C$, it was not unreasonable to suppose the difference in nutritional states among them did not exist. In the course of our experiment, it was observed that with prolonged acclimation at $14^{\circ}C$, some turtles did show symptoms which might have been attributed to nutritional deficiency. The eyelids of some of turtles developed a whitish membrane which gradually became swollen and death ensued some

time later. These turtles were not used. An explanation could be that at 14°C, the turtles were active enough to have appreciable metabolic demand, yet the temperature was too low to stimulate feeding so that in the long run, the animals could have suffered from a caloric deficiency. It is perhaps why data obtained from 14°C animals sometimes showed anomalous behaviour from the trend with temperature.

However, if there had been food intake deficiency in turtles at 14 and 5°C, both lung and surfactant lipids probably would have reflected it. This idea is supported by the results of a number of studies on the effect of fasting on mammalian lung summarized by Gross (1977). In all of these reports, if fasting had any effect, invariably changes occurred to PC in both lung tissue and lavage of the animal and in the same direction. When rats were fed a fat-free diet supplemented with 4% (w/w) of tripalmitoyl glycerol to incur a deficiency in essential fatty acids, it took 14 weeks to achieve the deficiency. Again, the results indicated a significant reduction of palmitate in both lung tissue and lavage fluid of the essential fatty acid-deficient group, with a corresponding increase in palmitoleate and oleate contents in PC of both lung and surfactant. However, no difference was observed in the total phospholipid content in lung tissue and surfactant in the essential fatty acid deficient group and the control (Kyriakides et al., 1976), which is different from our findings (Tables, 1,6,7).

In view of these observations, we feel that the feeding habits of the turtles were not a major factor in the changing profile of lipids

in surfactant. To definitively answer the question about food intake, however, an experiment in which turtles kept at 32°C are starved is being carried out to see if the phospholipid or fatty acid profiles of surfactant or lung are being affected.

(d) Oxygen uptake: For ectothermic animals, the rate of metabolism increases with environmental temperature. The feeding behaviour of turtles at different temperatures was indicative of this fact. It is therefore expected that the rate of oxygen uptake is higher for turtles at higher temperatures. This has been verified by a number of reports (Jackson *et al.*, 1974; see e.g. Howell and Rahn, 1976; Reeves, 1977). In turtles, oxygen consumption increased from 0.24 at 10°C to 0.88 $\frac{\text{ml STPD}}{\text{Kg min.}}$ at 35°C (Jackson, 1971). (STPD denotes the conditions at which oxygen is being measured, which is at standard temperature and pressure and the gas is dry). He also estimated the oxygen extraction coefficient, the fraction of inspired oxygen which the animal utilized, which he found to increase linearly with temperature. Only 6.8% of the inspired O₂ was utilized at 10°C, whereas more than 50% was consumed at 35°C. It is possible that the increase in the concentration of surfactant phospholipids at higher temperatures as observed in our study might be a response directed to meet this requirement, that is, that the oxygen extraction becomes more efficient as concentration of surfactant lipid increases. In a report on oxygen solubilization of lung surfactant, (Stanaszek *et al.*, 1976) the authors were able to show that appreciable oxygen gas absorption occurred in concentrated dog lung surfactant, and it occurred only to a small degree in dilute lung surfactant in saline. Although they did

not have results for oxygen absorption with increased surfactant concentrations, they suggested that the solubilization of oxygen might be dependent on the degree of what they called "micelle development" in the surfactant solution, which in turn depends on the concentration of the surfactant. Such a concentration dependence, however, would again not explain the changed nature of the surfactant lipids. A detailed study on oxygen solubility in aqueous dispersion of DPPC has indicated that, among other things, both in the gel and liquid crystalline state, the bilayers as a whole dissolve oxygen to a considerable extent. However, the solubility of oxygen in the lamellae when the temperature is below the phase transition, that is, when the DPPC bilayer is in the gel state, is about the same as water. Above the transition, when DPPC is in liquid crystalline state, the solubility is 3-4 times higher (Peters and Kimmich, 1978). Thus these changes would not appear to be adaptation for gaseous exchange in turtle lung. The more compelling explanation for the alteration of lipid profiles in turtle surfactant with changing temperature was that of a direct response to the temperature change itself, so that surfactant lipid remained in a unique physical state(s) at any given temperature. In other words, homeoviscous adaption occurred in lung surfactant. More direct evidence in favour of this argument will be presented below.

2. Fulfillment of Homeoviscous Adaption in Surface Activity and Film Spreading

The all important function of lung surfactant is the lowering of surface tension in the small alveoli to a greater extent than in the bigger ones, so that collapse of the lung during expiration is prevented. It is generally accepted that in mammals DPPC is the component

responsible; DPPC, in the form of films, has been shown to lower surface tension to zero under certain experimental conditions (Caliccico and Scarpelli, 1970, 1973). There is morphological evidence that the alveolar surface film of mammals is pure lipid (Kaibara and Kikkawa, 1971; Kikkawa, 1975). In vitro the close similarity of the thermal transition curves of crude lung wash and of extracted lipid also suggested that protein in lung surfactant has little influence on lipid-lipid interactions (Träuble et al., 1974). Direct measurement of alveolar surface tension has made possible the in situ experimentation on surfactant function which led Clements to postulate that during inflation, the alveolar surface film is like purified lung surfactant, but becomes mainly DPPC during deflation (Clements, 1977). The lowering of surface tension to 9 mN/m or less in the small alveoli depends on the close packing of DPPC film beyond its equilibrium value of 24-28 mN/m (Schürch et al., 1976).

It has been calculated that in mammals, DPPC can cover the alveolar area 3-7 times (Clements et al., 1970), but estimates based on our results, showed that only at 32°C, was there enough DPPC to cover the entire turtle lung alveoli once. The results are shown in the Table below.

Temperature °C	Number of times DPPC can cover entire area	Number of times PC can cover entire alveoli
5	0.4	3.0
14	0.2	2.0
22	0.8	4.3
32	1.2	5.7

- Assumptions: (1) Area of turtle lung = 214 cm^2 per gm of fresh tissue (Clements et al., 1970).
- (2) Limiting area of DPPC = 44A^2 per molecule (Phillips and Chapman, 1968; Träuble et al., 1974).
- (3) Values in column C of Table 22a were used for DPPC.

It is noted that the surface active material in the report of Clements et al. (1970) included the intracellular component of surfactant but ours included only the extracellular material in the lavage. It can be argued that if the functional residual capacity (FRC) of turtle lung is 40% of the maximum volume as in mammals, and since lung area is directly proportional to volume (Boyle et al., 1977), DPPC could still cover the entire lung area at FRC, except for turtles at 14°C and perhaps the version of mechanism proposed by Clements (1977) for mammalian lung could also apply to turtles; that is, during lung inflation, the alveolar lining is made up of heterogenous mixture of lipids, but during lung deflation, the alveoli are stabilized by almost DPPC alone through lowering of surface tension. The higher DPPC content at higher temperatures could be said to be consistent with the demand for rapid replacement of the escaped surfactant during high compression. It has been calculated that by observing the increase in tension at constant areas in vitro the rate of escape of DPPC or other phospholipids at low surface tension (high compression) increases 5 fold for each increase of 10°C (Clements, 1977).

However, the DPPC content in surfactant estimated for turtles at 14°C was too low even to cover the area of lung at FRC, even though the turtles at 14°C might have produced anomalous results for reasons discussed above. If one considers the view that DPPC may not be the

only lipid capable of achieving an adequate reduction in surface tension at the appropriate temperature, these results may not be unusual (see below).

The Table (p. 130) also shows if other molecular species of PC were included to cover the alveoli, then there would be enough material to do so, even for turtles at 14°C. In fact, the results of our experiments tend to suggest that perhaps, it is the physical state or phase(s) of lung surfactant which is important for function, and that DPPC at 37°C is predominantly important only because its physical properties are singularly suitable to meet the requirement of the phase of surfactant at that temperature.

After being secreted into the hypophase, the fluid covering the alveolar wall, lung surfactant has to adsorb to the interface before it can function to lower surface tension. The in situ experiments in mammalian lung have demonstrated that adsorption occurs within a few minutes ($k' \approx 0.5 \text{ min.}^{-1}$) and during high compression, the surfactant is very stable. At FRC, alveolar surface tension approaches zero and increases very slowly at constant lung volume ($k' \approx 0.02 \text{ min.}^{-1}$) (Clements, 1977). The following evidence suggests that both of these processes might be phase dependent: (a) DPPC in its pure form, is an utter failure at adsorption onto the interface at 37°C or less, whereas purified surfactant which contains some unsaturated lipids forms surface films in a few minutes only, at low subphase concentration. (King and Clements, 1972c). That this process is not very sensitive to temperature, could be explained if the purified surfactant is sufficiently fluid at 10°C, so that by increasing temperature to 45°C, the rate constants for absorption would therefore hardly change.

(b) With fully saturated lipids, like DPPC, the rate of escape of the surface film, as shown by increase of tension from a very low value at constant area is very low ($K' < 0.006 \text{ min.}^{-1}$ at 37°C). When the films contain unsaturated lipids, the rates are much higher ($K' > 1.0 \text{ min.}^{-1}$ at 37°C). Unlike the process of adsorption this is highly temperature dependent; 5 fold increase for each increase of 10°C , for phospholipids containing 2 acyl groups whose absolute rate may differ by 2 orders of magnitude at a given temperature (Clements, 1967).

If these two processes were phase-dependent, it follows that the overall physical state specific for each temperature would be reflected by the composition of surfactant lipids peculiar to that temperature and that the changes with temperature would be such that the functional state should be preserved at all temperatures.

Our findings are consistent with this interpretation. It can be seen that at lower temperature, molecular species other than DPPC may be important in the functioning as surfactant. This perhaps also explains why there is such high quantity of DPPC in mammals whose body temperature is 37°C .

If spreading, or adsorption were dependent upon only the monomer concentration, it would be unlikely that the process is phase-dependent. It would seem, however, that since the critical micellar concentration of lecithin is so low ($\sim 10^{-10} \text{ M}$) (Tanford, 1973 pp. 100) that the monomer concentration should be independent of total lipid concentration. The concentration dependence found by King and Clements (1972c) would suggest a more complex method of insertion. The insertion of a multilayered unit of surfactant lipid into the interface as suggested by Morley et al. (1978) can more easily

be seen to be phase-dependent. Our findings of changing composition in response to temperature would not be inconsistent with this or any other aggregate transfer model.

There is other in vitro evidence to show that the lowering of surface tension is phase-dependent. In addition to the monolayer studies (van Deenen, 1962; Phillips and Chapman, 1968), dimyristoyl lecithin (DMPC) films produced minimum surface tension values of 22 mN/m at both 25°C and 37°C (Colacicco et al., 1976). DMPC has a transition temperature at 23.7°C (Phillips et al., 1969) and at the temperature measured, it is in liquid-crystalline phase and hence too expanded to achieve low surface tension. DPPC films lower surface tension to zero at both temperatures, as its transition is at 41°C. However, at 37°C, DPPC films are more expanded than at 25°C. The surface tension-area curves of distearyol lecithin (DSPC) films at 37°C are similar to those of DPPC and DSPC at 25°C. This, as the authors pointed out, is because both 37 and 25°C are far below the transition temperature of DSPC which is 58°C (Phillips et al., 1969).

An exciting observation has been made which relates the function of lung surfactant to the phase transition of the phospholipid film at the interface of alveoli (Träuble et al., 1974). Based on the occurrence of phase transitions in the alveolar surfactant and the presence of extended plateaus in the surface pressure-area isotherms of the extracted lipids, they proposed that alveoli show critical behaviour at physiological temperatures, i.e. by decreasing the transpulmonary pressure during exhalation, a critical point will be reached so that further small decrease in pressure would produce large volume changes. With this abrupt decrease in volume, and therefore, area of the surface,

the lipid structure switches from fluid to condensed state and stabilizes the small alveoli by lowering the surface tension appreciably and can result in reopening of the alveoli. They also suggest that the concomitant phase transition might help to regulate gas transport across the alveolar film. Although the argument is somewhat weakened by subsequent findings that pure DPPC surface films below the transition temperature are in arbitrary metastable states when surface pressure exceeds about 0.1 mN/m and the film properties are dependent on the way in which the film is spread (Horn and Gershfeld, 1977), the thesis that phase transitions of lung surfactant may be critical for function remains an intriguing one.

In passing, it is interesting to note that Pattle reported bubbles from the lungs of Rana temporaria are less stable than those from Xenopus laevis at room temperature, but they "click" at 3°C (Pattle, 1976). "Clicking" and the stability ratio are phenomena attributable to the occurrence of surface active film of the bubble (Pattle, 1965). He did not report the temperature at which the animals were kept, but based on our results, it is tempting to speculate that the frogs (Rana temporaria) might have been kept at low temperatures and were able to produce lung surfactant suitable to function only at that temperature.

Also, there is a report describing pulmonary lavage from bullfrogs (Rana catesbeiana). At room temperature this was able to lower surface tension to only 19.5 mN/m (compared to 12 mN/m or lower for mammals). The bullfrogs had been kept at 4°C for 2 weeks (Hitchcock and Parsons, 1977). The question to ask is: Had the surface tension measurement been carried out at 4°C, would a value comparable to that of mammalian lung surfactant

have been obtained?

Obviously, the next step to take in the verification of the importance of thermal phase transition in lung surfactant is to carry out surface tension studies using lavage obtained from different temperature groups and compare the minimum surface tension measured at its environmental temperature with that measured at other temperatures.

3. Lipid Synthesis

Our results showed that with rising body temperature increases were found in the total lipid of lavage fluid per gram of lung tissue and in the proportion of DPPC in the PC. It is not possible to say at this time whether this represented an increase in the synthesis or the secretion of these components. It would seem reasonable to assume in the light of homeoviscous adaptation in other species that it is the phospholipid synthetic pathways which were responding to temperature.

The major route for the de novo synthesis of mammalian PC in surfactant appears to be via cytidine diphosphorylcholine (see e.g. Frosolono, 1977; Mason 1976). Although this pathway is not known to be selective for any particular species of lecithin but 1-saturated, 2-unsaturated ones, recent work using mammalian type II cells in culture (Smith and Kikkawa, 1978) has shown that the PC is retailored to DPPC via the deacylation-reacylation pathway of Lands (1960). There is no reason to assume that the same pathways do not operate in the turtles and that perhaps one or both are responsive to body temperature. If the turtle is found to produce DPPC and other lecithin species in surfactant by the same routes as those in mammals, then the turtles could serve as a useful animal model for the study of surfactant lipid synthesis since it is responsive to the simply-controlled condition of environmental

temperature.

Summary and Conclusions

The effect of temperature on the lipid composition of lung surfactant in map turtles has been examined. The changes were compared with those occurring in lung tissue.

Map turtles, Malaclemys geographica, were acclimated for at least one month to 5, 14, 22 and 32°C before they were utilized after euthanasia. The turtle lung was lavaged in situ using physiological saline to obtain the surface active material from the alveolar wall. Cell count of the supernatant indicated that contamination of lipid from lung cells sloughed off during lavage was negligible. Lipid was extracted for subsequent analyses.

Lipid phosphorus was found to increase in surfactant but not in lung when the environmental and body temperature was increased. This general increase in phospholipid concentration in surfactant with increasing temperature was consistent with the enhanced efficiency in oxygen uptake by the animal at high temperatures, among other functions, since solubilization of oxygen has been demonstrated to be dependent on the concentration of phospholipid in surfactant.

Phospholipid composition of lung surfactant of turtles resembled that of mammalian surfactant in that phosphatidylcholine constituted more than 70% of phospholipid in both, suggesting a similarity in the vital functions of lung surfactant in mammals and turtle. Phosphatidylcholine and phosphatidylinositol were the two phospholipids largely responsible for the increase in lipid phosphorus in surfactant over 5-32°C. This result coupled with the fact that they were the two major lipids in surfactant tend to lend weight to the argument that phosphatidylcholine

and phosphatidylinositol might play a more prominent role in the functioning of lung surfactant in turtles. Changes in phosphatidylcholine of lung tissue was accompanied by opposite changes in phosphatidylethanolamine with temperature. This might reflect a requirement for a constant amount of lipid zwitterionic head groups in lung membranes.

The fatty acid profile of total lipid in lung consisted of oleate, palmitate, arachidonate, stearate and palmitoleate as the major components. A peak with retention time similar to that of cholesterol was present in total lipid of lung. In contrast, the major fatty acids in total lipid of surfactant were palmitate, oleate, palmitoleate and stearate. There was a significant increase in palmitate and decrease in palmitoleate content of total lipids in surfactant with increasing temperature. The nonsystematic changes with temperature occurring to total lipids in lung probably underscored the diversity of cell types which have a wide range of functions.

Palmitate comprised more than 50 mole % of total fatty acids of phosphatidyl choline in surfactant where a significant enrichment of palmitate and palmitoleate was observed, compared with the corresponding phospholipid in lung. The changes in the fatty acids of surfactant with temperature were distinct from those occurring to the fatty acids of phosphatidylcholine of lung tissue.

Palmitoleate of phosphatidylinositol in surfactant decreased with increasing temperature while no significant change was exhibited in the corresponding lipid of lung. These data were in harmony with the generally accepted scheme of mammalian surfactant in that phosphatidylcholine was the major surface active component, and phosphatidyl-

inositol, analogous to phosphatidylglycerol in the mammalian system, was functionally important. Further, the results indicated that the functioning of the surfactant system may be temperature sensitive, and implies a need for constancy in the physical state of surfactant.

Phosphatidylcholine of lung and surfactant, and phosphatidylethanolamine of lung showed an increase in saturation with increasing temperature. These observations were consistent with the maintenance of a specific phase at each temperature in surfactant and to some extent in lung cells. To achieve this, the quantity of DPPC in surfactant was regulated, as evidenced by the increase with increasing temperature in the saturated phosphatidylcholine isolated which consisted of predominantly dipalmitoyl phosphatidylcholine. The remarkable quantity of diunsaturated phosphatidylethanolamine of lung, on the other hand, leads one to speculate that the regulation of the production of diunsaturated phospholipid could be a possible mechanism for the lung cell to maintain a definite physical state with changes in temperature.

It was expected that these changes in surfactant would result in changes of their physical state, based on the knowledge derived from monolayer and bilayer studies. This was confirmed by the differential scanning calorimetric observations on some model mixed lecithins in water.

Changes in environmental temperature induce changes in the respiratory physiology of the ectotherm. The possible effect of these temperature-dependent variables on lung surfactant were discussed and it is argued, in light of the whole body of evidence presented here, that the most cogent explanation for the changes observed in the surfactant lipids was that the physical state of lung surfactant lipid was critical for function. Hence map turtles adapted themselves to a given temperature by changing the lipid composition to preserve the specific physical state

at a given temperature. The conclusion of our study was that homeoviscous adaptation occurred in turtle lung surfactant.

References

- Abe, M., Ohno, K. and Sato, R. (1974). *Biochim. Biophys. Acta* 369, 361-370.
- Abrams, M.E. (1966). *J. Appl. Physiol.* 21, 718-720.
- Akino, T., Abe, M. and Arai, T. (1971). *Biochim. Biophys. Acta* 248, 274-281.
- Artom, C. (1968). *Fed. Proc.* 27, 457.
- Askin, F.B. and Kuhn, C. (1971). *Lab. Invest.* 25, 260-268.
- Avery, M.E. and Fletcher, B.D. (1974). in The Lung and Its Disorders in the Newborn Infant, 3rd Ed., ed. Schaffer, A.J., W.B. Saunders Co., Philadelphia, London, Toronto, 10-15, 220.
- Avery, M.E. and Mead, J. (1959). *Am. J. Dis. Child.* 97, 517-523.
- Avery, M.E. and Said, S. (1965). *Medicine* 44, 503-526.
- Avery, M.E., Wang, N.S. and Taeusch, H.W., Jr. (1973). *Sci. Am.* 228, 75-85.
- Ballard, P.L. (1977). in Development of the Lung, ed. Hodson, W.A. Marcel Dekker Inc., New York, 419-442.
- Ballard, P.L. and Ballard, R.A. (1972). *Proc. Natl. Acad. Sci. USA* 69, 2668-2672.
- Ballard, P.L., Benson, B.J. and Brehier, A. (1977). *Am. Rev. Respir. Dis.* 115, (Supplement), 29-35.
- Bartlett, G.R. (1959). *J. Biol. Chem.* 234, 466-468.
- Bennett, A.F. (1972). *J. Comp. Physiol.* 79, 259-280.
- Bennett, A.F. (1973). *Comp. Biochem. Physiol.* 46A, 673-690.
- Bligh, E.G. and Dyer, W.J. (1959). *Can. J. Biochem. Physiol.* 37, 911-917.
- Boyle, J., III., Englestein, E.S. and Sinoway, L.J. (1977). *Respiration* 34, 241-249.
- Bremer, J. and Greenberg, D.M. (1959). *Biochim. Biophys. Acta (Amst)* 35, 241-249.
- Brett, J.R. (1972). *Respir. Physiol.* 14, 151-170.
- Brooks, R.E. (1970). *Z. Zellforsch.* 106, 484-497.

- Brown, E.S. (1964). *Am. J. Physiol.* 207, 402-406.
- Brown, E.S., Johnson, R.P. and Clements, J.A. (1959). *J. Appl. Physiol.* 14, 717-720.
- Buckingham, S., Heinemann, H.O., Sommers, S.C. and McNary, W.F. (1966). *Am. J. Pathol.* 48, 1027-1041.
- Buckingham, S., McNary, W.F. and Sommers, S.C. (1964). *Science* 145, 1192-1193.
- Chapman, D. (1973). in Form and Function of Phospholipids, ed. Ansell, G.B., Hawthorne, J.N. and Dawson, R.M.C. Elsevier Scientific Publishing Co., Amsterdam, London, New York, 117-142.
- Chapman, D. (1975). *Quart. Rev. Biophys.* 8, 185-235.
- Chapman, D., Urbina, J. and Keough, K.M. (1974). *J. Biol. Chem.* 249, 2512-2521.
- Chapman, D., Williams, R.M. and Ladbroke, B.D. (1967). *Chem. Phys. Lipid* 1, 445-475.
- Chevalier, G. and Collet, A.J. (1972). *Anat. Rec.* 174, 289-310.
- Claus, M.H., Clements, J.A. and Harel, R.J. (1961). *Proc. Natl. Acad. Sci. USA* 47, 1858-1859.
- Clements, J.A. (1957). *Proc. Soc. Exp. Biol. Med.* 95, 170-172.
- Clements, J.A. (1962). *Sci. Amer.* 207, 120-130.
- Clements, J.A. (1967). in Development of the Lung, ed. de Reuck, A.V.S. and Porter, R., J. and A. Churchill, London, 202.
- Clements, J.A. (1977). *Am. Rev. Respir. Dis.* 115 (Supplement), 67-71.
- Clements, J.A., Brown, E.S. and Johnson, R.P. (1958). *J. Appl. Physiol.* 12, 262-268.
- Clements, J.A., Hustead, R.F., Johnson, R.P. and Gribetz, I. (1961). *J. Appl. Physiol.* 16, 444-450.
- Clements, J.A. and King, R.J. (1976). in The Biochemical Basis of Pulmonary Function, ed. Crystal, R.G., Marcel Dekker Inc., New York and Basel, 363-387.
- Clements, J.A., Nellenbogen, J. and Trahan, H.J. (1970). *Science* 169, 603-604.
- Clements, J.A. and Tierney, D.F. (1965). in Handbook of Physiology Section IV, Respiration Volume II, ed. Fenn, W.O. and Rahn, H., Am. Physiol. Society, Chapter 69, 1565-1583.

- Clements, J.A. and Trahan, H.J. (1963). Fed. Proc. 22, 281.
- Colaccico, G., Basu, M.K. and Scarpelli, E.M. (1976). Respir. Physiol. 27, 169-186.
- Colaccico, G., Buckelew, R., Jr. and Scarpelli, E.M. (1973). J. Appl. Physiol. 34, 743-749.
- Colaccico, G., Ray, A.F. and Buckelew, A.R., Jr. (1977). Lipid 12, 879-888.
- Colaccico, G. and Scarpelli, E.M. (1970). Fed. Proc. 29, 661.
- Colaccico, G. and Scarpelli, E.M. (1973). in Biological Horizons in Surface Science, ed. Prince, L.M. and Sears, D.F., Academic Press, New York, 367-425.
- Comroe, J.H., Jr. (1965). Physiology of Respiration Year Book, Medical Publishers, Chicago.
- Cossins, A.R. (1977). Biochim. Biophys. Acta 470, 395-411.
- Cossins, A.R. and Prosser, C.L. (1978). Proc. Natl. Acad. Sci. 75, 2040-2043.
- Crawford, E.C. and Schultetus, R.R. (1970). Copeia 1970, 179-180.
- Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1969). in Data for Biochemical Research, 2nd Ed., Oxford, 508.
- Dejours, P., Garey, W.F. and Rahn, H. (1970). Respir. Physiol. 9, 108-117.
- Dermer, G.B. (1969). J. Ultrastrut. Res. 27, 88-104.
- Erbland, J.F. and Marinetti, G.V. (1965). Biochim. Biophys. Acta 106, 128-138.
- Farrell, P.M. (1973). in Respiratory Distress Syndrome, ed. Villee, C.A., Villee, D.B. and Zuckerman, J., Academic Press, New York, 311-341.
- Farrell, P.M. and Blackburn, W.R. (1973). Pediatr. Res. 7, 308.
- Farrell, P.M., Lundgren, D.W. and Adams, A.H. (1974). Biochim. Biophys. Res. Commun. 57, 696-700.
- Farrell, P.M. and Morgan, T.E. (1977). in Development of the Lung, ed. Hodson, W.A., Marcel Dekker Inc., New York and Basel, 309-347.
- Franson, R., Beckerdite, S., Wang, P., Waite, M. and Elsbach, P. (1973). Biochim. Biophys. Acta 296, 365-373.

- Frosolono, M.F. (1977). in Lipid Metabolism in Mammals, Volume 2, ed. Synder, F., Plenum Press, New York and London, 1-58.
- Frosolono, M.F., Charms, B.C. and Pawlowski, R. (1970). J. Lipid Res. 11, 439-457.
- Frosolono, M.F., Pawlowski, R. and Charms, B.C. (1975). Chest 67, 16s-19s.
- Fung, Y.C. (1975). Circ. Res. 37, 497-502.
- Galdston, M., Shah, D.O. and Shinowara, G.Y. (1969). J. Colloid Interface Sci. 29, 319-334.
- Garcia, A., Newbirk, J.D. and Mairs, R.D. (1975). Biochem. Biophys. Res. Commun. 64, 128-135.
- Gassenheimer, L.N. and Rhoades, R.A. (1974). J. Appl. Physiol. 37, 224-227.
- Gassenheimer, L.N., Rhoades, R.A. and Scholz, R.W. (1972). Respir. Physiol. 15, 268-275.
- Gaunt, A.S. and Gans, C. (1969). J. Morph. 128, 195-218.
- Giannapoulos, G., Mulay, S. and Solomon, S. (1972). Biochem. Biophys. Res. Commun. 47, 411.
- Gil, J. and Reiss, O.K. (1973). J. Cell. Biol. 58, 152-171.
- Gluck, L., Kulovich, M.V., Eidelman, A.E., Cordero, L. and Khazin, A.F. (1972). Pediatr. Res. 6, 81-89.
- Gluck, L., Motoyama, E.K., Smits, H.L. and Kulovich, M.V. (1967). Pediatr. Res. 1, 237-246.
- Godinez, R.I., Sanders, R.L. and Longmore, W.J. (1975). Biochemistry 14, 830-834.
- Goerke, J. (1974). Biochim. Biophys. Acta 344, 241-261.
- Coerke, J. and Clements, J.A. (1973). Physiologist 16, 323.
- Graham, J.B. (1974). Respir. Physiol. 21, 1-7.
- Gribetz, I., Frank, N.R. and Avery, M.E. (1959). J. Clin. Invest. 38, 2168.
- Gross, J. (1977). Fed. Proc. 36, 2665-2669.
- Gross, I. and Warshaw, J.B. (1974). Pediatr. Res. 8, 193-199.
- Gruenwald, P. (1963). Lab. Invest. 12, 563-576.

- Hallman, M. and Gluck, L. (1976). J. Lipid. Res. 17, 257-262.
- Harlan, W.R., Jr., Margraf, J.H. and Said, S.I. (1966). Am. J. Physiol. 211, 855-861.
- Henderson, R.F. and Clayton, M.H. (1976). Anal. Biochem. 70, 440-446.
- Henderson, R.F. and Pflieger, R.C. (1972). Lipid 7, 492-494.
- Hendry, A.T. and Possmayer, F. (1974). Biochim. Biophys. Acta 369, 156-172.
- Hitchcock, K.R. and Parsons, W.J. (1977). J. Histochem. Cytochem. 25, 1363-1367.
- Hochachka, P.W. and Somero, G.N. (1973). in Strategies of Biochemical Adaptation, W.B. Saunders Company, Philadelphia, London, Toronto, 212-216.
- Holub, B.J. and Lands, W.E. (1975). Can. J. Biochem. 53, 1262-1277.
- Horn, L.W. and Gershfeld, N.L. (1977). Biophys. J. 18, 301-310.
- Howell, B.J. and Rahn, H. (1976). in Biology of the Reptilia, Vol. 5, Physiology A, ed. Gans, C. and Dawson, W.R., Academic Press, London, 335-363.
- Hurst, D.J., Kilburn, K.H. and Lynn, W.S. (1973). Respir. Physiol. 17, 72-80.
- Jackson, D.C. (1971). Respir. Physiol. 12, 131-140.
- Jackson, D.C., Palmer, S.E. and Meadow, W.L. (1974). Respir. Physiol. 20, 131-146.
- James, A.T. and Martin, A.J.P. (1952). Biochem. J. 50, 679-690.
- Jobe, A. (1977). Biochim. Biophys. Acta 489, 440-453.
- Kaibara, M. and Kikkawa, Y. (1971). Am. J. Anat. 132, 61-77.
- Kates, M. (1972). in Techniques of Lipidology; Isolation, Analyses and Identification of Lipids, North-Holland Publishing Company, 437-438.
- Kennedy, E.P. and Weiss, S.B. (1956). J. Biol. Chem. 222, 193-214.
- Kikkawa, Y. (1975). in Pulmonary Physiology of the Fetus, Newborn and Child, ed. Scarpelli, E.M. and Auld, P.A.M., Lea and Febiger, Philadelphia, 37-60.
- Kikkawa, Y. and Yoneda, G.K. (1974). Lab. Invest. 30, 76-84.
- King, R.J. (1977). Am. Rev. Respir. Dis. 115 (Supplement), 73-79.

- King, R.J. and Clements, J.A. (1972a). Am. J. Physiol. 223, 704-714.
- King, R.J. and Clements, J.A. (1972b). Am. J. Physiol. 223, 715-726.
- King, R.J. and Clements, J.A. (1972c). Am. J. Physiol. 223, 727-733.
- King, R.J., Gikas, E.G., Rush, J. and Clements, J.A. (1974). Am. Rev. Respir. Dis. 110, 273-281.
- King, R.J., Klass, D.J., Gikas, E.G. and Clements, J.A. (1973). Am. J. Physiol. 224, 788-795.
- King, R.J., Rush, J., Gikas, E.G., Platzker, A.C.G. and Creasy, Z.K. (1975). J. Appl. Physiol. 39, 735-741.
- Klaus, M.H., Clements, J.A. and Havel, R.J. (1961). Proc. Natl. Acad. Sci. USA 47, 1858-1859.
- Klaus, M.H., Reiss, O.K., Tooley, W.H., Piel, C. and Clements, J.A. (1962). Science 137, 750-751.
- Kito, M. (1977). Biochem. (Japan) 49, 1301-1315.
- Kotas, R.V. and Avery, M.E. (1971). J. Appl. Physiol. 30, 358-361.
- Krogh, A. (1941). in The Comparative Physiology of Respiratory Mechanisms, University of Pennsylvania Press, Philadelphia.
- Kuenzig, M.C., Hamilton, R.W., Jr. and Peltier, I.F. (1965). J. Appl. Physiol. 20, 779-782.
- Kyriakides, E.C., Beeler, D.A., Edmonds, R.H. and Balint, J.A. (1976). Biochim. Biophys. Acta 431, 399-407.
- Lampert, J. and Macklem, P.T. (1971). J. Appl. Physiol. 31, 380-385.
- Lands, W.E.M. (1958). J. Biol. Chem. 231, 883-888.
- Lands, W.E.M. (1960). J. Biol. Chem. 235, 2233-2237.
- Laplace, P.S. de. (1807). Traité de Macanique Céleste Tome IV.
- Lee, A.G. (1977a). Biochim. Biophys. Acta 472, 237-281.
- Lee, A.G. (1977b). Biochim. Biophys. Acta 472, 285-344.
- Liggins, G. (1969). J. Endocrinol. 45, 515-523.
- Longmore, W.J. and Mourning, J.T. (1977). J. Lipid Res. 18, 309-313.
- Lucey, E.C. and House, E.W. (1977). Comp. Biochem. Physiol. 57A, 239-243.

- Macklin, C.C. (1954). *Lancet* 266, 1099-1104.
- Maguire, J.J., Shelley, S.A., Paciga, J.E. and Balis, J.U. (1977). *Prepar. Biochem.* 7, 415-425.
- Marten, F. (1671). quoted in: Scammon, C.M. (1874), *The Marine Mammals of the North-Western Coast of North America*, 196, Carmany & Co., San Francisco, reprinted (1968) by Dover, New York.
- Mason, R.J. (1976). in *The Biochemical Basis of Pulmonary Function*, ed. Crystal, R.G., Marcel Dekker, Inc., New York and Basel, 127-169.
- Mead, J. (1961). *Physiol. Rev.* 41, 281-330.
- Meban, C. (1972). *J. Cell. Biol.* 53, 249-252.
- Meyer, F. and Bloch, K. (1963). *J. Biol. Chem.* 238, 2654-2659.
- McElhaney, R.N. and Souza, K.A. (1976). *Biochim. Biophys. Acta* 443, 348-359.
- Miller, D.A. and Bondurant, S. (1961). *J. Appl. Physiol.* 16, 1075-1077.
- Mims, L.C. and Kotas, R.V. (1973). *Biol. Neonate* 22, 436-443.
- Mims, L.C. and Zee, P. (1971). *Biol. Neonate* 18, 356-362.
- Morgan, T.E. (1969). *Biochim. Biophys. Acta (Amst.)* 178, 21-34.
- Morgan, T.E., Finley, T.N. and Fialkow, H. (1965). *Biochim. Biophys. Acta* 106, 403-413.
- Moriya, T. and Kanoh, H. (1974). *Tohoku. J. Exp. Med.* 112, 241-256.
- Morley, C.J., Bangham, A.D., Johnson, P., Thornburn, G.D. and Jenkin, G. (1978). *Nature* 271, 162-163.
- Myrvik, Q.N., Leake, E.S. and Fariss, B. (1961a). *J. Immunol.* 86, 128-132.
- Myrvik, Q.N., Leake, E.S. and Fariss, B. (1961b). *J. Immunol.* 86, 133-136.
- Naimark, A. (1973). *Fed. Proc.* 32, 1967-1971.
- Notter, R.H. and Morrow, P.E. (1975). *Ann. Biomedic. Engineering* 3, 119-159.
- Okada, Y., Ishiko, S., Daido, S., Kim, J. and Ikeda, S. (1962). *Acta Tuberc. Jap.* 12, 1-10.
- Okano, G. and Akino, T. (1978). *Biochim. Biophys. Acta* 528, 373-384.

- Oyarzun, M.J. and Clements, J.A. (1977). *J. Appl. Physiol: Respir., Environ., Exercise Physiol.* 43, 39-45.
- Papahadjopoulos, D. (1973). in *Form and Function of Phospholipids*, ed. Ansell, G.B., Dawson, R.M.C. and Hawthorne, J.N., Elsevier Scientific Publishing Company, Amsterdam, 143-169.
- Passero, M.A., Bhattacharyya, S.N. and Lynn, W.S. (1973). *Proc. Natl. Acad. Sci.* 70, 973-976.
- Passero, M.A., Bhattacharyya, S.N. and Lynn, W.S. (1974). *Clin. Res.* 22, 571A.
- Pattle, R.E. (1955). *Nature* 175, 1125-1126.
- Pattle, R.E. (1958). *Proc. Roy. Soc. Lond.* B148, 217-240.
- Pattle, R.E. (1965). *Physiol. Rev.* 45, 48-79.
- Pattle, R.E. (1976). in *Respiration of Amphibious Vertebrates*, ed. Hughes, G.M., Academic Press, London, 233-255.
- Pattle, R.E. and Hopkinson, D.A.W. (1963). *Nature* 200, 894.
- Paul, G.W., Hassett, R.J. and Reiss, O.K. (1977). *Proc. Natl. Acad. Sci.* 74, 3617-3620.
- Pawlowski, R., Frosolono, M.F., Charms, B.L. and Przybylski, R. (1971). *J. Lipid Res.* 12, 538-544.
- Peters, A. and Kimmich, R. (1978). *Biophys. Struct. Mechanism* 4, 67-85.
- Pfeger, R.C., Henderson, R.F. and Waide, J. (1972). *Chem. Phys. Lipids* 9, 51-68.
- Pfeger, R.C. and Thomas, H.G. (1971). *Arch. Intern. Med.* 127, 863-872.
- Phillips, M.C. and Chapman, D. (1968). *Biochim. Biophys. Acta* 163, 301-313.
- Phillips, M.C., Williams, R.M. and Chapman, D. (1969). *Chem. Phys. Lipids* 3, 234-244.
- Pitkin, R.B. (1977). *Comp. Biochem. Physiol.* 57A, 413-416.
- Platzker, A.C., Kitterman, J.A., Mescher, E.J., Clements, J.A. and Tooley, W.H. (1975). *Pediatr.* 56, 554-561.
- Popjak, G. and Beekmans, M. (1950). *Biochem. J.* 47, 233-238.
- Possmayer, F., Duwe, G., Hahn, M. and Buchnea, D. (1977a). *Can. J. Biochem.* 55, 609-617.

- Prosser, C.L. (1961). in Comparative Animal Physiology, 2nd Ed. ed. Prosser, C.L. and Brown, F.A.J., Saunders, Philadelphia and London.
- Pruitt, K.M., Cherng, M.J. and Spitzer, H.L. (1971). Arch. Intern. Med. 9, 6-10.
- Radford, E.P. (1954). Proc. Soc. Exp. Biol. Med. 87, 58-63.
- Radford, E.P., Jr., Lefcoe, N. and Mead, J. (1954). Fed. Proc. 13, 114-115.
- Radford, E.P., Jr. and McLaughlin, M. (1956). Fed. Proc. 15, 147.
- Reeves, R.B. (1977). Ann. Rev. Physiol. 39, 559-586.
- Reifenrath, R. (1975). Respir. Physiol. 24, 115-137.
- Rooney, S.A. and Wai-Lee, T.S. (1977). Lung 154, 201-211.
- Roth, J., Winkelmann, H. and Meyer, A.W. (1973). Exp. Path. 8, 354-362.
- Sanders, R.L. and Longmore, W.J. (1975). Biochemistry 14, 835-840.
- Sanderson, R.J. and Vatter, A.E. (1977). J. Cell. Biol. 74, 1027-1031.
- Sawada, H., Yamabayashi, H. and Okajima, Y. (1977). Biochem. Biophys. Acta 493, 196-200.
- Scarpelli, E.M. (1968). The Surfactant System of the Lung, ed. Lea Febiger, Philadelphia.
- Scarpelli, E.M., Clutario, B.C. and Taylor, F.A. (1967). J. Appl. Physiol. 23, 880-886.
- Scarpelli, E.M. and Colacicco, G. (1970). Advan. Exp. Biol. Med. 7, 275-286.
- Scarpelli, E.M., Condorelli, S., Colacicco, G. and Cosmi, E.V. (1975). Pediatr. Res. 9, 195-201.
- Schiller, H. and Bensch, K. (1971). J. Lipid Res. 12, 248-255.
- Schurch, S., Goerke, J. and Clements, J.A. (1976). Proc. Natl. Acad. Sci. USA 73, 4698-4702.
- Shelley, S.A., L'Heureux, M.V. and Balis, J.U. (1975). J. Lipid Res. 16, 224-234.
- Shelley, S.A., Paciga, J.E. and Balis, J.U. (1977). Lipid 12, 505-510.
- Sinensky, M. (1974). Proc. Natl. Acad. Sci. 71, 522-525.

- Skipski, V.P. and Barclay, M. (1969). in Methods in Enzymology, Vol. XIV, ed. Lowenstein, J.M., Academic Press, London, 530-598.
- Smith, F.B. and Kikkawa, Y. (1978). Lab. Invest. 38, 45-51.
- Smith, M.W. (1967). Biochem. J. 105, 65-71.
- Smith, M.W. (1976). in Biochemical Adaptation to Environmental Change, ed. Smellie, R.M.S. and Pennock, J.F., Biochem. Soc., London, 43-60.
- Snyder, F. and Malone, B. (1975). Biochem. Biophys. Res. Commun. 66, 914-919.
- Sorokin, S.P. (1970). in Morphology of Experimental Respiratory Carcinogenesis, ed. Nettesheim, P., Hanna, M.G., Jr. and Deatherage, J.W., Jr., Oak Ridge, U.S. Atomic Energy Commission Technical Information Extension, 3-43.
- Spitzer, H.L. and Norman, J.R. (1971). Arch. Intern. Med. 127, 429-435.
- Spitzer, H.L., Rice, J.M., MacDonald, P.C. and Johnston, J.M. (1975). Biochem. Biophys. Res. Commun. 66, 17-23.
- Stanaszek, W.F., Ecanow, B. and Levinson, R.S. (1976). J. Pharmaceutical Sci. 65, 142-143.
- Standaert, T. and Johansen, K. (1974). J. Comp. Physiol. 89, 313-320.
- Steim, J.M., Redding, R.A., Hauck, C.T. and Constantine, H. (1969a). Fed. Proc. 28, 400.
- Steim, J.M., Reinert, J.C., Tourtellotte, M.E., Rader, R.L. and McElhaney, R.N. (1969b). Proc. Natl. Acad. Sci. USA 63, 104-109.
- Strang, L.B. (1977). Am. Rev. Physiol. 39, 253-276.
- Sueishi, K., Tanaka, K. and Oda, T. (1977). Lab. Invest. 37, 136-142.
- Summer, J.L. and Morgan, E.D. (1969). J. Gen. Microbiol. 59, 215-221.
- Swan, H. (1974). in Thermoregulation and Bioenergetics, American Elsevier Publishing Company Inc., New York, London, Amsterdam, 121-125.
- Tabak, S.A. and Notter, R.H. (1975). Fed. Proc. 34, 426.
- Tabak, S.A., Notter, R.H., Ultman, J.S. and Dinh, S.M. (1977). J. Colloid Interface Sci. 60, 117-125.
- Taeusch, H.W., Jr. and Avery, M.E. (1977). in Development of the Lung, ed. Hodson, A.W., Marcel Dekker Inc., New York and Basel, 399-418.

- Tanford, C. (1973). in The Hydrophobic Effect, John Wiley & Sons, Inc. New York, London, Sydney, Toronto, 100.
- Tenney, S.M. and Remmers, J.E. (1963). *Nature* 197, 54-56.
- Tenney, S.M. and Tenney, J.B. (1970). *Respir. Physiol.* 9, 197-215.
- Thannhauser, S.J., Benotti, J. and Boncoddio, N.F. (1946). *J. Biol. Chem.* 166, 669-675.
- Thomas, T. and Rhoades, R.A. (1970). *Am. J. Physiol.* 219, 1535-1538.
- Tierney, D.F. (1965). *Dis. Chest* 47, 247.
- Tierney, D.F. (1974). *Ann. Rev. Physiol.* 36, 209-231.
- Tierney, D.F., Clements, J.A. and Trahan, H.J. (1967). *Am. J. Physiol.* 20, 1253-1260.
- Tierney, D.F. and Johnson, R.P. (1961). *Physiologist* 4, 122.
- Tierney, D.F. and Johnson, R.P. (1965). *J. Appl. Physiol.* 20, 1253-1260.
- Tombropoulos, E.G. (1964). *Science* 146, 1180-1181.
- Toshima, N. and Akino, T. (1972). *Tohoku J. Exp. Med.* 108, 253-263.
- Toshima, N., Akino, T. and Ohno, K. (1972). *Tohoku J. Exp. Med.* 108, 265-277.
- Träuble, H., Eibl, H. and Sawada, H. (1974). *Naturwissenschaften* 61, 344-354.
- Tsao, F.H.C. and Zachman, R.D. (1977). *Pediatr. Res.* 11, 858-861.
- van Deenen, L.L.M. (1966). *Ann. N.Y., Acad. Sci.* 137, 717-730.
- van Deenen, L.L.M., Houtsmuller, U.M.T., de Haas, G.H. and Mulder, E. (1962). *J. Pharm. Pharmacol.* 14, 429-444.
- Vaughan, D.J. and Keough, K.M. (1974). *FEBS Letters* 47, 158-161.
- von Neergaard, K. (1929). *Z. Gesamte. Exp. Med.* 66, 373-394.
- Vereyken, J.M., Monfoort, A. and van Golde, L.M.G. (1972). *Biochim. Biophys. Acta* 260, 70-81.
- Webster, G.R. (1965). *Biochim. Biophys. Acta* 98, 512-519.
- Weibel, E.R. (1967). in Development of the Lung, ed. de Reuck, A.V.S. and Porter, R., Churchill, London, 170-176.

- Weibel, E.R. (1973). *Physiol. Rev.* 53, 419-495.
- Weibel, E.R., Gehr, P., Haris, D., Gil. J. and Bachofen, M. (1976).
in Lung Cells in Disease, ed. Bouhuys, A., Amsterdam, North-
Holland, 3.
- Weibel, E.R. and Gil. J. (1968). *Respir. Physiol.* 4, 42-57.
- Weinhold, P.A., Skinner, R.S. and Sanders, R.D. (1973). *Biochim.*
Biophys. Acta 326, 43-51.
- Weinhold, P.A. and Villee, C.A. (1965). *Biochim. Biophys. Acta* 106,
540-550.
- Wood, S.C. and Lenfant, C.J.M. (1976). in Biology of the Reptilia,
Vol. 5, Physiology A, ed. Gans, C. and Dawson, W.R., Academic Press,
London, 225-274.
- Wyszogrodski, I., Kyei-aboagye, K., Taeusch, H.W., Jr. and Avery, M.E.
(1975). *J. Appl. Physiol.* 38, 461-466.
- Zachman, R.D. (1972). *Biol. Neonate.* 19, 211-219.



